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Adenosine production inside rat polymorphonuclear leucocytes

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Adenosine synthesis was studied during 2-deoxyglucose-induced ATP catabolism in intact rat polymorphonuclear leucocytes. When both adenosine kinase (EC 2.7.1.20) and adenosine deaminase (EC 3.5.4.4) were selectively inhibited, adenosine accumulated. Adenosine formation took place inside the intact cells by a metabolic pathway independent of the ecto-5′-nucleotidase (EC 3.1.3.5). Distinct metabolic pathways are proposed for adenosine production from intracellular or extracellular nucleotides.

Adenosine has been implicated as a regulatory metabolite in such diverse processes as coronary blood-flow regulation (Berne, 1964), fat-cell lipolysis (Schwabe *et al.*, 1973), neurotransmitter release (Phillis *et al.*, 1979) and platelet aggregation (Haslam *et al.*, 1978). Despite considerable progress in understanding the mechanism of action of adenosine (Londos *et al.*, 1978) the mechanism and control of adenosine production remain the subject of conjecture and hypothesis (Berne, 1980; Arch & Newsholme, 1978). The enzyme generally assumed to be responsible, the plasma-membrane 5′-nucleotidase, has been shown to be an ecto-enzyme (i.e. to have its catalytic site on the external membrane face; De Pierre & Karnovsky, 1974; Newby *et al.*, 1975) and furthermore to be unessential for the hydrolysis of intracellularly generated IMP (Newby, 1980). However, its role in adenosine production from AMP has remained unclear. At least in the synaptic cleft or during platelet aggregation, adenosine may arise by the action of the ecto-enzyme on extracellular AMP (Dowdall, 1978; Pearson *et al.*, 1980). However, in the perfused heart, evidence has been presented for intracellular adenosine synthesis (Frick & Lowenstein, 1976). In view of the cellular complexity and multiple compartments in the perfused organ, it was essential to examine this question in an intact isolated cell. A further complication in studying adenosine production has been the ubiquitous presence of adenosine-metabolizing enzymes. This was circumvented in one study (Matsumoto *et al.*, 1979) by using a mutant lymphoblastoid cell line which lacked the enzyme adenosine kinase (EC 2.7.1.20). Adenosine deaminase (EC 3.5.4.4) was inhibited, as in the present work, with 2′-deoxycoformycin. The characterization of adenosine kinase inhibitors which are effective on the enzyme in intact cells (Newby, 1981) opens up the possibility of extending such studies to a wide variety of normal cells. In the first application of this strategy, we have established unequivocally that adenosine may be synthesized within an intact cell by a pathway independent of the ecto-5′-nucleotidase. We propose the existence of distinct metabolic pathways for the generation of adenosine from intracellular and extracellular nucleotides.

Experimental

2′-Deoxycoformycin {*R*-3-(2-deoxy-β-D-*erythro*-pentofuranosyl) - 3,6,7,8 - tetrahydro[4,5 - *d*][1,3]diazapin-8-ol} and 5-iodotubercidin {4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine} were generously given by Dr. H. W. Dion, Parke–Davis, Detroit, MI, U.S.A., and Dr. Leroy B. Townsend, University of Michigan College of Pharmacy, Ann Arbor, MI, U.S.A., respectively.

Rat polymorphonuclear leucocytes were obtained 98% pure and incubated in Krebs– Ringer bicarbonate buffer as described previously (Newby, 1980). Termination of incubations with trichloroacetic acid and processing of samples for high-pressure liquid chromatography were also described there. Metabolite standards added immediately after trichloroacetic acid precipitation were completely recovered and did not suffer conversion into detectable amounts of any other metabolite measured.

Determination of metabolites was improved by a slight modification of the previously published chromatography method (Newby, 1980). Elution at 2.0ml/min with 50mM-$(NH_4)_2HPO_4$, pH6.25, gave

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the following retention times (min): IMP, 3.1; ATP, 3.5; ADP, 4.2; hypoxanthine, 5.8, AMP, 6.7; inosine, 13.2. Elution at 2.0ml/min with 50mM-$(NH_4)_2HPO_4$, pH7.0, containing 10% (v/v) methanol gave the following retention times (min): nucleotides and hypoxanthine, <4.0; *S*-adenosylhomocysteine, 6.3; adenosine, 9.2; 2′-deoxyadenosine, 12.0. In each case absorbance peaks were completely resolved. The identification of absorption peaks corresponding to metabolites in cell extracts was as described previously (Newby, 1980), except for adenosine. This was identified by collecting the column effluent (from approx. 10^7 cells) containing the peak with retention time 9.2min. The effluent was free from 2′-deoxycoformycin and hence could be treated with adenosine deaminase (1 unit for 10min at 37°C). On rechromatography, no peak corresponding to adenosine was found, but a quantitatively equivalent peak with retention time 3.6min corresponding to inosine was found.

Results and discussion

Catabolism of adenine nucleotides was induced by incubation in the absence of glucose with 5mM-2-deoxyglucose, an analogue which may be phosphorylated but not further metabolized. Rapid catabolism of ATP (74±4%, paired results from 11 experiments) resulted in 2min at 37°C (Table 1). This was followed by a slower decline to 14.9±0.7% (paired results from 26 experiments) by 10min. Over this period lactate dehydrogenase (EC 1.1.1.27) release from the cells, an assay of cell breakage, rose from 0.93±0.07% (30) to 1.28±0.13% (22) of the homogenate value. ADP concentration increased by approx. 50% at 2min, but then declined to control values at 10min (Table 1). AMP concentration, on the other hand, rose about 5-fold and remained elevated to 10min. IMP concentration showed an initial dramatic increase, followed by a slower fall. Inosine and hypoxanthine accumulated as the end-products of nucleotide catabolism (Table 1), provided that an inhibitor of xanthine oxidase (EC 1.2.3.2) was present. Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine; 10μM) was maximally effective and had no effect on any other metabolite concentrations measured (results not shown). These observations indicated that a low activity of xanthine oxidase (<0.5nmol/min per 10^7 cells; Newby, 1980) is present in polymorphonuclear leucocytes. The accumulation of hypoxanthine as the end-product of cyanide-induced adenine nucleotide catabolism (Newby, 1980) is explained by the known inhibitory effect of cyanide on xanthine oxidase (Bray, 1975).

It may be noted from Table 1 that the sum of the concentrations of ATP and its catabolites after treatment of cells for 10min with 2-deoxyglucose (14.1±0.4nmol/10^7 cells) exceeded that of control cells (10.7±0.3nmol/10^7 cells). This suggests that metabolites other than ATP contributed to the rate of catabolite formation. Interconversion of other purine nucleotides and deoxyribonucleotides (Murray *et al.*, 1970) and synthesis of IMP *de novo* are possible sources of such intermediates.

Cells incubated without inhibitors of adenosine deaminase or adenosine kinase did not show a measurable accumulation of adenosine in either the presence or absence of 2-deoxyglucose (Table 1). To prevent metabolism of adenosine, cells were incubated with 5-iodotubercidin, an inhibitor of adenosine kinase (Wotring & Townsend, 1979; Newby, 1981) in combination with 2′-deoxycoformycin, a tight-binding inhibitor of adenosine

Table 1. *Metabolite concentrations and cell breakage*

Values of cellular metabolite concentrations (except for adenosine) and supernatant lactate dehydrogenase were pooled from experiments either with or without inhibitors of adenosine-metabolizing enzymes or of ecto-phosphatases. Values throughout this paper are expressed as means±S.E.M. for the numbers of separate experiments shown in parentheses.

Conditions	Metabolite concn. (nmol/10^7 cells)							Lactate dehydrogenase release (% of homogenate activity)
	ATP	ADP	AMP	IMP	Adenosine	Inosine	Hypoxanthine	
Control	8.3	1.31	0.28	0.46	0.043	0.09	0.21	0.93
	±0.3	±0.07	±0.03	±0.06	±0.007	±0.01	±0.03	±0.07
	(42)	(42)	(33)	(14)	(14)	(5)	(32)	(30)
+2-deoxyglucose (2min)	2.4	2.0	1.3	3.6	0.027	1.1	1.0	—
	±0.1	±0.2	±0.1	±0.4	±0.004	±0.2	±0.1	
	(9)	(14)	(19)	(12)	(5)	(6)	(14)	
+2-deoxyglucose (10min)	1.31	1.12	1.0	2.3	0.036	1.35	7.0	1.3
	±0.07	±0.06	±0.1	±0.4	±0.007	±0.06	±0.05	±0.1
	(27)	(29)	(23)	(9)	(9)	(9)	(27)	(22)

deaminase (Agarwal *et al.*, 1977). The inhibitors were used at concentrations previously shown maximally to inhibit their target enzymes *in situ* in intact polymorphonuclear leucocytes (Newby, 1980, 1981).

On addition of 2-deoxyglucose, adenosine accumulated rapidly to 0.33 ± 0.04 (7) nmol/10^7 cells at 2 min and then more slowly to 0.52 ± 0.06 (23) nmol/10^7 cells at 10 min (Fig. 1). Inhibition of adenosine kinase alone did not lead to accumulation of adenosine (results not shown), whereas inhibition of the deaminase only led to a sub-maximal and transient accumulation (Fig. 1).

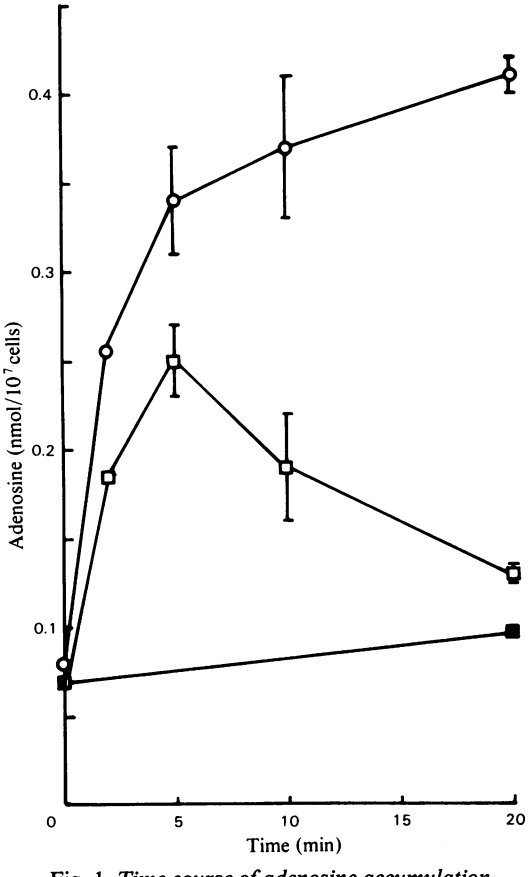

Fig. 1 *Time course of adenosine accumulation*
Polymorphonuclear leucocytes ($0.60 \pm 0.02 \times 10^7$/ml) were preincubated in duplicate at 37°C for 30 min with either 30 μM-2′-deoxycoformycin + 1 μM-5-iodotubercidin (○, ■) or 30 μM-2′-deoxycoformycin alone (□). At 0 min, 5 mM-2-deoxyglucose was added (○, □) or alternatively incubation continued in the absence of this agent (■). Samples were taken at the times shown and adenosine concentration was determined by high-pressure liquid chromatography. Values are the means and range for one of four similar experiments.

The increase in adenosine concentration at 10 min was equivalent to $5.9 \pm 0.6\%$ (8) of the fall in ATP concentration and was too small to be reflected in a statistically significant change of any other metabolite (results not shown). These data confirm that degradation of AMP to adenosine is a minor catabolic route in these cells, most of the hypoxanthine arising from IMP and inosine (Newby, 1980).

To determine whether adenosine was formed within the polymorphonuclear leucocytes, cells were rapidly separated from their surrounding medium. The amount of adenosine present in the supernatant was compared with that in the whole cell suspension (Fig. 2). The amount of adenosine in the cell pellet is not recorded, since the considerable time and harsh conditions needed for its resuspension resulted in additional adenosine synthesis. The data show that only 11% of the adenosine synthesized in response to 2-deoxyglucose was present in the medium at 2 min. This value increased to 19% at 5 min. At this cell dilution 95% of the total water was extracellular. It was therefore concluded that adenosine was synthesized inside the cells and a proportion was subsequently transported into the medium.

A combination of inhibitory antibodies raised to rat liver ecto-5′-nucleotidase (Stanley *et al.*, 1980) and 10 mM-β-glycerophosphate inhibited the ecto-phosphatases of rat polymorphonuclear leucocytes by 98% (Newby, 1980). When cells so inhibited were treated with deoxyglucose, the same changes in metabolite concentrations occurred as in control cells (results not shown). In particular, the rate of hypoxanthine accumulation recorded in three time-course experiments was 0.65, 0.75 and 1.35 nmol/min per 10^7 cells. The residual ecto-phosphatase activity in the presence of inhibitors was 0.14, 0.17 and 0.15 nmol/min per 10^7 cells respectively. This data amplified our previous conclusion that catabolism of IMP to hypoxanthine did not require ecto-phosphatases (Newby, 1980).

To test the effect of antibodies and β-glycerophosphate on adenosine synthesis directly, cells were incubated in the simultaneous presence of these agents and the inhibitors of adenosine metabolism. Since at 37°C the rate of adenosine production might already be slowed at the first 2 min time-point (Figs. 1 and 2), these experiments were conducted at 25°C. Fig. 3 shows that the maximal rate of adenosine synthesis, measured between 2 and 5 min was unaffected by 98% inhibition of ecto-phosphatase activity. This suggested that intracellular adenosine synthesis had a distinct enzymic mechanism.

Further experiments will be necessary to establish the nature of this mechanism. Several mammalian cells (Stanley *et al.*, 1980), including polymorphonuclear leucocytes (Newby, 1980), contain an

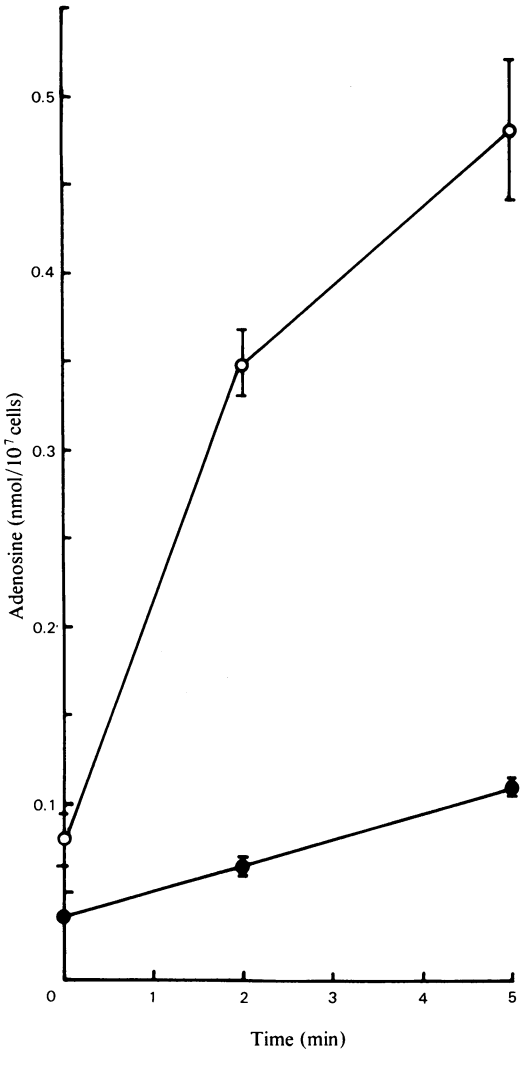

Fig. 2. *Adenosine accumulation in cells and suspending medium*

Polymorphonuclear leucocytes ($1.78 \pm 0.05 \times 10^7$/ml) were preincubated in groups of eight at 37°C for 30min with 30μM-2′-deoxycoformycin + 1μM-5-iodotubercidin. At 0min, 5mM-2-deoxyglucose was added. At the times shown trichloroacetic acid (final concn. 5%, w/v) was added to four samples, and the remaining suspensions were centrifuged for 5s at 15000rev./min in a bench-top Microfuge (Eppendorf model 5412) in the presence of a 1:1 (v/v) mixture of silicone oils DC 550 and DC 556 (Newby, 1980). The supernatant, which was separated from the cells by the oil layer, was collected into trichloroacetic acid within 15s. There was no detectable loss of adenosine from a supernatant of polymorphonuclear leucocytes containing 2′-deoxycoformycin in 1h (results not shown). Values of adenosine concentration in the supernatant (●) or in the whole cell suspension (○) are means ± S.E.M. for one of four similar experiments.

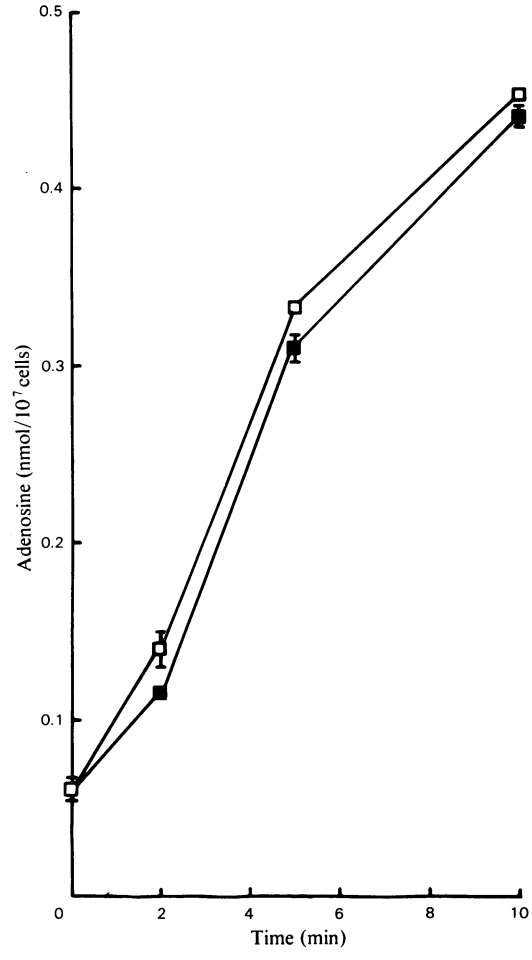

Fig. 3. *Independence of adenosine accumulation from the activity of ecto-phosphatases*

Polymorphonuclear leucocytes ($1.57 \pm 0.02 \times 10^7$/ml) were pretreated in triplicate as in the legend to Fig. 2, with the addition of 1/100 dilution of sheep anti-(rat liver 5′-nucleotidase) serum (■). They were then cooled to 25°C, and at 0min 5mM-deoxyglucose (□) or 5mM-2-deoxyglucose + 10mM-sodium β-glycerophosphate (■) (final concns.) was added. After the times shown incubations were terminated and adenosine concentrations determined. Values are the means and ranges from one of three similar experiments. Separate quadruplicate samples of the same cells were assayed for ecto-phosphatases by method 3 of Newby *et al.* (1975). Total ecto-AMPase activity determined at 25°C was for control cells 3.6 ± 0.2nmol/min per 10^7 cells and for inhibited cells 0.06 ± 0.006nmol/min per 10^7 cells.

internal pool of the ecto-enzyme, although this is thought to be confined to the intravesicular surface of subcellular vesicles (Stanley *et al.*, 1980). A

cytoplasmic 5′-nucleotidase has been detected in liver (Fritzon, 1969) and also in polymorphonuclear leucocytes (Newby, 1980). Its possible role in adenosine production should be tested. A third possibility is that adenosine arises from *S*-adenosylhomocysteine (Schrader *et al.*, 1981). We view this as an unlikely explanation for our results, since the concentration of this metabolite remained unaltered and below 0.06 nmol/10^7 cells under all the conditions of the present study. There is also no established mechanism for increased synthesis of *S*-adenosylhomocysteine during energy depletion.

Catabolism of extracellular AMP by ecto-5′-nucleotidase can explain the generation of adenosine in the synaptic cleft and during platelet aggregation (Dowdall, 1978; Pearson *et al.*, 1980). The present work demonstrates an alternative route for intracellular adenosine production. The similarities in this process in polymorphonuclear leucocytes and in the perfused heart (Frick & Lowenstein, 1976) encourage the belief that this mechanism is physiologically important. In both cell types, the rate of adenosine production appears to be low in the normal state (Fig. 1), but on energy depletion a prompt and pronounced acceleration takes place. The strategy outlined above, by greatly exaggerating the accumulation of adenosine in a non-perfused system, permits its easy determination by the currently available methods. Also, by allowing the measurement of adenosine production in the absence of degradation, it should greatly facilitate the study of the mechanism and control of this important regulatory process.

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