THE ADENOSINE-LIKE EFFECT OF EXOGENOUS CYCLIC AMP UPON NUCLEOTIDE AND PP-RIBOSE-P CONCENTRATIONS OF CULTURED HUMAN LYMPHOBLASTS

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1. Introduction

cAMP and dbcAMP produce opposite metabolic effects when added to cultures of mammalian cells [1–5]. For example, cAMP increased the glycogen content of HeLa cells whereas dbcAMP caused a reduction [1,3]. These differences are due apparently to the extracellular catabolism of cAMP and the resistance of dbcAMP to degradation. The increase in cellular glycogen could be produced by other adenosine phosphates and was not due to increase of intracellular cAMP concentration [3,6,7]. Extracellular catabolism of cAMP pointed to adenosine being the active metabolite [2,3,6,8]. Exogenously added cAMP or adenosine increases cellular ATP concentration [3,9,10], the rate of uridine [2,3,5,8] and thymidine [2,3] incorporation into nucleic acids and reduces UTP concentrations [2,3,11].

The present study demonstrates the catabolism of cAMP to adenosine by two serums commonly used in tissue culture. Exogenous cAMP added to lymphoblasts in culture, in acting as a precursor of adenosine, reduced PP-ribose-P concentrations and thereby caused a depletion of pyrimidine nucleotides and NAD and inhibited cell growth. The mechanism of adenosine-mediated lymphoblast toxicity may be relevant to understanding the relationship between the immunodeficiency disease associated with human adenosine deaminase deficiency [12].

Abbreviations: cAMP, adenosine 3′,5′- cyclic phosphate; dbcAMP, $N^6O^{2'}$-dibutyryl adenosine 3′,5′-cyclic phosphate; PP-ribose-P, 5-phosphorylribose 1-pyrophosphate; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; EHNA, erythro-9(2-hydroxy-3-nonyl) adenine.

2. Materials and methods

2.1. *Materials*

Radiochemicals were purchased from Amersham Searle: [8-^{14}C]adenine, 59 mCi/mmole; [8-^{14}C] adenosine, 59 mCi/mmole; and [8-^{3}H]cAMP, 27 Ci/mmole. Adenosine, AMP, cAMP, cGMP, PP-ribose-P, and D-ribose-5-phosphate were purchased from P & L Biochemicals; dbcAMP from CalBiochem. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was provided by Dr H. J. Schaeffer, then at Wellcome Research Laboratories, North Carolina.

WI-L2 human lymphoblasts and an HGPRT-deficient subline [13] were grown in autoclavable minimum essential medium with 2 mM glutamine and 10% horse serum (Flow Laboratories) and maintained between 10^4 and 10^6 cells per ml. Cell counts were measured using a Coulter counter, model Z_{B1}.

2.2. *Measurement of cellular nucleotide and orotic acid concentrations*

Acid-soluble nucleotides were separated on a Varian LCS-1000 high-pressure liquid chromatograph equipped with a 3-meter capillary column packed with Varian PA-38 anion exchange resin; the nucleotide elution profile was essentially as previously described [14,15]. Peak areas were determined by height multiplied by width at half height and compared to a series of known standards. AMP and IMP have coincident retention times and their area has been arbitrarily ascribed solely to AMP. Orotic acid also has a retention time nearly coincident with AMP and was therefore quantitated by subjecting samples to hydrolysis in 1 N HCl at 100°C for 60 min or con-

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verting the orotic acid to OMP by incubation of neutralized extracts with partially purified yeast orotate phosphoribosyltransferase (Sigma) and PP-ribose-P prior to nucleotide analysis. Cells were harvested and extracted for analysis as previously described [15].

2.3. *Assay of cAMP and adenosine metabolism*

After incubation of [^{3}H]cAMP or [^{14}C]adenosine in medium containing 10% horse or fetal calf serum for various times, 0.050 ml aliquots were added to tubes containing 0.010 ml 8 M formic acid, and 0.025 ml were spotted on cellulose thin layers (Eastman Kodak) for separation of cAMP, AMP, and adenosine in isopropyl alcohol–concentrated ammonia–water (7:1:2) [16]; or nucleotides, inosine, hypoxanthine, adenosine, adenine, in *n*-butanol–methanol–water–ammonia (60:20:20:1) [17]. Separated compounds were cut out and counted [17].

2.4. *Assay of intracellular PP-ribose-P concentration and PP-ribose-P synthetase activity*

PP-ribose-P concentrations were measured by a modification of a previously reported method [8].

PP-ribose-P synthetase (EC 2.7.6.1) activity was measured on the day of harvesting in parent and HGPRT deficient lymphoblast lysates after centrifugation, 20 000 *g*, and passage of supernatant through Sephadex G10. Order of substrate additions were important in this assay (R. C. Willis, personal communication) as modified from a previous method [19]; assays were started by simultaneous addition of 5 mM ATP and 65 μM [^{14}C]adenine, after 10 min preincubation of enzyme and 1 mM ribose-5-phosphate.

3. Results

3.1. *Catabolism of cAMP in horse and fetal calf serum*

CAMP is converted to adenosine in medium containing 10% horse serum; the rate limiting step is conversion of cAMP to AMP (fig.1). The rates of cAMP catabolism (40 μM) in 10% horse and calf serum were 5.1 and 1.3 μmol/h, respectively. In medium containing 10% horse serum, the product adenosine accumulates because less than 5% of 50 μM adenosine is metabolized in 30 h at 37°C; this medium was used in all studies. Lymphoblast growth is inhibited more by cAMP than adenosine

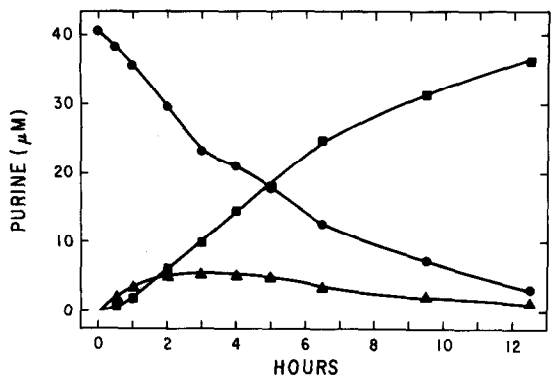

Fig.1. Metabolism of [^{3}H]cAMP in medium containing 10% horse serum at 37°C. cAMP (●), AMP (▲), adenosine (■).

at 50 μM, because the adenosine slowly generated from cAMP is not as effectively deaminated by cellular adenosine deaminase, having a K_M of approx. 30 μM [20,21], as a single addition of adenosine.

3.2. *Effect of cAMP on lymphoblast nucleotide and orotic acid concentrations*

50 μM dbcAMP had little effect on growth rate whereas 50 μM cAMP produced transient growth inhibition (2–3 days, fig.2) and 500 μM cAMP arrested growth and ultimately caused cell death.

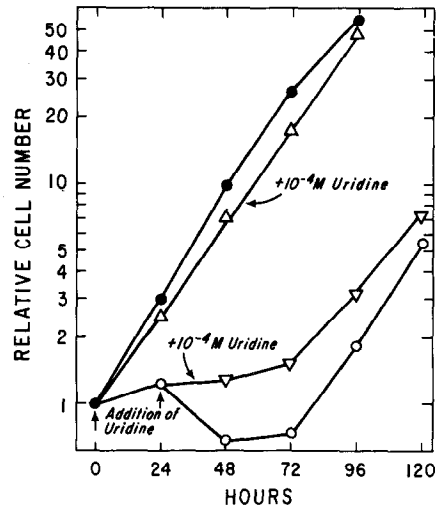

Fig.2. Inhibition of WI-L2 lymphoblast growth by cAMP and reversal by uridine. Lymphoblasts were cultured in medium containing 10% horse serum with no additions (●); 50 μM cAMP (○); 50 μM cAMP with 100 μM uridine added at 0 h (△), or 24 h (▽).

Table 1
Effect of 10 h incubation with 500 μM cAMP upon nucleotide pools of human lymphoblasts

Nucleotide	Control (nmoles/10^6 cells) n=8	cAMP n=5	Ratio cAMP:control
AMP	0.25 ± 0.08	0.13* ± 0.03	
ADP	0.46 ± 0.13	0.66 ± 0.31	
ATP	4.79 ± 0.48	7.75 ± 3.11	
Total AXP	5.50	8.54	1.55
NAD	0.93 ± 0.06	0.48 ± 0.22	0.52
GMP	0.07 ± 0.04	–	
GDP	0.15 ± 0.05	0.18 ± 0.08	
GTP	1.21 ± 0.41	1.38 ± 0.63	
Total GXP	1.43	1.56	1.09
UDP	0.25 ± 0.12	0.05 ± 0.03	
UTP	1.96 ± 0.16	0.10 ± 0.04	0.05
CTP	0.79 ± 0.11	0.13 ± 0.04	0.17
UDP-sugars	1.23 ± 0.19	0.43 ± 0.27	0.35
Orotate	<0.02[a]	1.34[a] ± 0.14	
Adenylate energy charge	0.91	0.95	

Lymphoblasts in mid-log phase growth (2–5 X 10^5 cells/ml), were incubated in the presence or absence of 500 μM cAMP for 10 h at 37°C and the nucleotide concentrations determined by high pressure liquid chromatography. Results are the mean ± 1 S.D. of n experiments. The adenylate energy charge = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

[a] n=3

After a 10-h incubation of lymphoblasts with 500 μM cAMP, the NAD, UDP-sugars, and particularly UTP and CTP concentrations were greatly reduced; the concentration of ATP increased (table 1). There was no change or slight increase in the adenylate energy charge [22]. The cAMP, 500 μM, dependent decrease in UTP and CTP and increase in ATP were detectable at times as early as 2 or 4 h with maximal changes between 6 and 10 h.

During 10 h incubation with a range of cAMP concentrations (fig.3), as little as 10 μM cAMP decreased

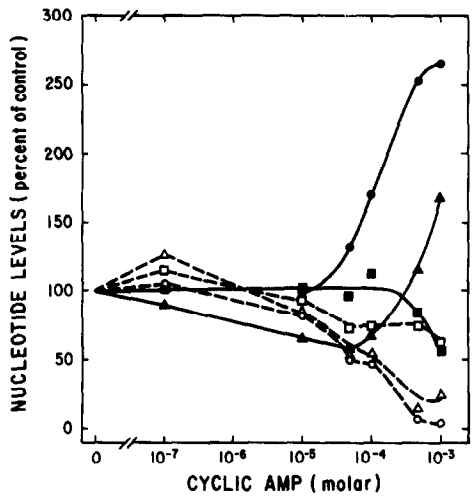

Fig.3. Effect of 10 h incubation with a range of cAMP concentrations on lymphoblast nucleotide concentrations. Percent of the control nucleotide concentrations given in table 1 for ATP (● – ●), GTP (▲ – ▲), NAD (■ – ■), UTP (○ - - ○), CTP (△ - - △), UDP-sugars (□ - - □).

pyrimidine nucleotide and GTP concentrations. The decrease in pyrimidine nucleotides and GTP were not related exclusively to an increase in ATP concentration. GTP returned to control or increased concentrations only at cAMP concentrations which resulted in increased ATP concentrations (fig.3).

The incubation of lymphoblasts with cAMP not only caused a gross depletion of pyrimidine nucleotides, but an accumulation of orotic acid (table 1). Addition of orotic acid, 500 μM, to lymphoblast cultures did not inhibit growth. Uridine, 100 μM, prevented or partially overcame the cAMP growth inhibition (fig.2), similar findings have been reported [11].

3.3. *Intracellular PP-ribose-P concentrations*

The depletion of NAD and pyrimidine nucleotides and particularly the accumulation of orotic acid, which requires PP-ribose-P for further metabolism, suggested the synthesis of PP-ribose-P may be inhibited. After 6 h exposure to 50 μM cAMP, AMP, or adenosine, intracellular PP-ribose-P concentrations were reduced by more than 80% (table 2). The greatest sustained depletion of PP-ribose-P at 24 h was produced by the combination of adenosine, 50 μM, and EHNA, 5 μM, an adenosine deaminase inhibitor [23] which inhibited lymphoblast adenosine deaminase activity by greater than 95%.

3.4. *Effect of cAMP and adenosine on PP-ribose-P synthetase activity*

The adenosine effect of reducing PP-ribose-P concentrations was apparently not mediated through inhibition of PP-ribose-P synthetase activity. The following compounds produced less than 10% change of PP-ribose-P synthetase activity in parent and HGPRT-deficient lymphoblast lysates: 500 μM adenosine, 5 μM EHNA, adenosine and EHNA in combination. The nucleotides, 100 μM cAMP or cGMP, also produced less than 5% change in PP-ribose-P synthetase activity at either 10^{-3} or 10^{-4} M ATP. PP-ribose-P synthetase activity was 2.9 nmol/mg protein per min.

4. Discussion

These results demonstrate the conversion of cAMP to adenosine in culture medium supplemented with either fetal calf or horse serum (fig.1). Addition of cAMP to human lymphoblasts in culture provided a precursor for the slow generation of adenosine, which resulted in a depletion of pyrimidine nucleotides and NAD and an accumulation of orotic acid (table 1). The depleted pyrimidine nucleotide pools account for the increased rates of labelled uridine [2,3,5,8] and thymidine [2,3] incorporation into nucleic acids of cAMP-adenosine treated cells.

Table 2
Effect of adenosine phosphates and adenosine on lymphoblast PP-ribose-P concentration

Additions	Intracellular PP-ribose-P (nmoles/10^9 cells)			
	6 h	Relative concentration	24 h	Relative concentration
None	175	1.00	244	1.00
Adenosine, 50 μM	28	0.16	47	0.19
AMP, 50 μM	16	0.09	33	0.14
cAMP, 50 μM	24	0.14	40	0.17
EHNA, 5 μM	155	0.89	199	0.81
EHNA, 5 μM + adenosine, 50 μM	39	0.22	16	0.07

Lymphoblasts in log phase growth were incubated with compounds for 6 or 24 h after which cells were extracted and PP-ribose-P concentrations were determined.

The reduction in cellular nucleotides and accumulation of orotic acid appear to be a consequence of reduced PP-ribose-P concentration (table 2). An inhibition of nucleotide and PP-ribose-P synthesis was not mediated by an imbalance in the adenylate energy charge (table 1). The low ATP:ADP ratio in a previous report, 1.7 for control, and 1.4 for adenosine treated cells [11] may reflect degradation during extraction or dead cells. An ATP:ADP ratio of 10 for adenosine treated cells, similar to the present results has also been reported [9]. Adenosine or cAMP also did not inhibit PP-ribose-P synthetase activity in lymphoblast lysates. Early addition of uridine overcame the cAMP-adenosine mediated growth inhibition (fig.2) and phosphorylation of uridine and adenosine could provide pyrimidine and purine nucleotides independent of PP-ribose-P. At varied cAMP concentrations GTP returned to normal or increased concentrations only as ATP became greater than control concentrations (fig.3), suggesting guanine nucleotides were derived from the expanding adenine nucleotide pool formed from adenosine.

Adenosine causes an increase in the glycogen content of cultured human cells [1,3] and has now been shown to decrease PP-ribose-P concentrations of human lymphoblasts. The purine analogs, 9-hydroxyalkyl- and 9-aminoalkyl purines, have been shown to inhibit the rate of PP-ribose-P dependent reactions and reduce the PP-ribose-P concentrations in tumor cells [24]. An adenosine analog which cannot be phosphorylated to the 5′-nucleotide, 2′,5′-dideoxyadenosine, also decreased the concentration of PP-ribose-P in mouse lymphoma cells [25]. The site(s) of action of these compounds and adenosine remain to be elucidated. The adenosine mediated increase in glycogen and decrease in PP-ribose-P concentrations suggest adenosine may cause an increase in glycogenesis and decrease in pentose phosphate synthesis.

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