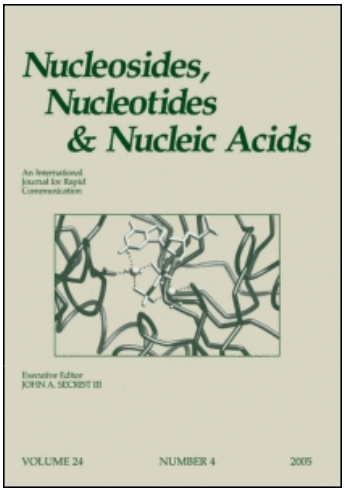

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Robbins, Brian L. , Greenhaw, Jack and Fridland, Arnold(2000) 'Cellular Phosphorylation of 2', 3'-Dideoxyadenosine-5'-monophosphate, a Key Intermediate in The Activation of the Antiviral Agent DDI, in Human Peripheral Blood Mononuclear Cells', Nucleosides, Nucleotides and Nucleic Acids, 19: 1, 405 — 413
To link to this Article: DOI: 10.1080/15257770008033017
URL: http://dx.doi.org/10.1080/15257770008033017

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(1&2), 405–413 (2000)

CELLULAR PHOSPHORYLATION OF 2', 3'-DIDEOXYADENOSINE-5'-MONOPHOSPHATE, A KEY INTERMEDIATE IN THE ACTIVATION OF THE ANTIVIRAL AGENT DDI, IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT

2',3'-dideoxyadenosine 5-monophosphate (ddAMP), is a key intermediate in the metabolism of the antiviral agent 2',3'-dideoxyinosine (ddI) to its active triphosphate derivative, 2',3'-dideoxyadenosine-5'-triphosphate (ddATP). The potential role of adenylate kinase in the phosphorylation of ddAMP was studied in human peripheral blood mononuclear cells (PBMC) and a human T cell line, CEMss. Subcellular distribution, sulfhydryl inhibitor, and substrate specificity studies support the hypothesis that the mitochondrial adenylate kinase (AK2) is a major route of cellular activation of these compounds in human lymphocytes.

2', 3'-Dideoxyinosine (ddI) is one of five nucleoside analogs approved for the treatment of AIDS. To be active, dideoxyinosine must be converted to dideoxyadenosine 5'-triphosphate. Dideoxyadenosine-5'-triphosphate (ddATP) is a potent chain terminating inhibitor of HIV reverse transcriptase and is considered the active metabolite responsible for the pharmacological activity of ddI [1-3]. The conversion of ddI to dideoxyadenosine-5'-monophosphate (ddAMP) is well documented involving the action of cytosolic 5' nucleotidase and adenylate succinate synthetase/lyase[4-6]; however, the enzymes responsible for the formation of ddATP from ddAMP are unclear. There are several enzymes that are known to catalyze the phosphorylation of ddAMP *in vitro*. These include adenylate kinase, CMP/UMP kinase (at low levels), and PRPP synthetase [7-11]. We have previously demonstrated that 9-(2-phosphonylmethoxyethyl)adenine (PMEA), an acyclic adenosine analog, is phosphorylated by the adenylate kinase isozyme type 2 (AK2) in the human lymphoid cell line CEMss [12]. In the present study we questioned if the same enzyme was responsible for ddAMP phosphorylation in PBMC, the primary target cells for HIV infection. Adenylate kinase, a ubiquitous enzyme exists in multiple forms in mammalian systems. Each differs in amino acid sequence, tissue distribution, and subcellular localization. A

cytosolic form, AK1, is the preponderant enzyme in skeletal muscle, erythrocytes, and brain. In other tissues such as liver, kidney, and heart, adenylate kinase is found within the intermembrane spaces of the mitochondria (AK2). A third isozyme, AK3, is found in the mitochondrial matrix of liver and heart and uses GTP as its primary phosphate donor [13-16]. The present study examined the subcellular distribution, sensitivity to sulfhydryl reactive compounds, substrate specificity, and kinetics of ddAMP phosphorylation in both activated and resting PBMC and compared them with CEMss cells.

MATERIALS AND METHODS

Chemicals. Dr. David Johns of the National Cancer Institute provided 2', 3' - dideoxyinosine. Radioactive [^{3}H]2', 3'-dideoxyinosine, [^{3}H]2', 3'-dideoxyadenosine-5'-monophosphate, and [^{3}H] 2'-deoxyadenosine monophosphate were purchased from Moravek Biochemicals, Brea, CA. Polyethylene imine-cellulose plates were purchased from Baxter Scientific Products (McGraw Park Ill.) and 2', 3'-dideoxyadenosine-5'-triphosphate was obtained from Pharmacia Biotech, Piscataway, NJ. 5,5'-dithio-bis(2-nitrobenzoate), p-hydroxy-mercuribenzoic acid, pyruvate kinase, phospho(enol) pyruvate, HEPES buffer, Trizma base, dithiothreitol, bovine serum albumin, phytohemagglutinin, ddATP, and dAMP were purchased from Sigma Chemical Co., St. Louis, MO. Media used for cell cultures were from Biowhittaker Inc., Walkersville, MD. Protease inhibitors obtained as a kit and interleukin-2 were purchased from Boehringer Mannheim Corp., Indianapolis, IN. Dideoxyadenosine monophosphate was produced by the addition of 1 mg of snake venom phosphodiesterase (Sigma Cat# . V 7000) to 100 µl of 5 mM ddATP for 2 h at room temperature. The protein was removed with a 10,000 molecular weight cut off Centricon concentrator and the purity assessed by HPLC.

Cells and cell fractionation. Peripheral blood mononuclear cells were separated from the whole blood of healthy donors by Ficoll hypaque density centrifugation. Whole blood was diluted 1:1 with Hank's buffered salt solution, layered onto lymphocyte separation media, and separated by centrifugation for 30 min at 400 x g. The mononuclear layer was removed, washed once with growth media, counted, and pelleted by centrifugation. The cells were suspended in growth media and the residual erythrocytes lysed by the addition of 3 volumes of cold water for 30 s. The lysis was stopped by the addition of 1 volume of 600 mM NaCl. The PBMCs were pelleted by centrifugation, suspended in growth media and transferred to plastic tissue culture flasks. Adherent cells were removed and the cells were adjusted to a density of 1 million cells per ml. Stimulated PBMCs were produced by incubation for 72 h with 5 µg/ml of PHA, 10 units/ml interleukin 2, 25 mg/ml penicillin/streptomycin, and 1x non essential amino acids. If quiescent cells were required the PHA and interleukin 2 were omitted and the cells used the following day. The cells were fractionated as previously described [12] and kinase activities assessed.

CEMss cells were obtained from Peter Nara through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. Cells in log phase were collected by centrifugation, washed twice with phosphate buffered saline, and separated into mitochondrial, cytosolic, and microsomal fractions [12].

Nucleotide monophosphate kinase assays. Assays were performed to determine the phosphorylation of AMP, UMP, dAMP, and ddAMP. Activities were determined at 37 °C in a reaction volume of 50 μl containing 50 mM Tris buffer (pH 7.0), 5 mM $MgCl_2$, 3 mM dithiothreitol, 15 mM phospho(enol) pyruvate, 0.5 μg of bovine serum albumin per ml, 2 mM ATP, and 1 unit of pyruvate kinase. The protein concentrations for AMP and UMP kinase determinations were 0.1 μg and 1 μg of mitochondrial and cytosolic preparation per reaction, respectively. The reactions with dAMP and ddAMP were not as efficient, requiring 1 and 10 μg of mitochondrial and cytosolic preparation per reaction. The incubations were initiated by the addition of the appropriate radioactive substrate: [^{3}H] AMP, [^{3}H] UMP, [^{3}H] dAMP, and [^{3}H]ddAMP at concentrations of 50 μM and an activity of 0.3 Ci/mol. The reactions were terminated after 30 min by the addition of 10 μl of 0.3 M ethylenediamine tetra-acetic acid and 4 μl aliquots were spotted onto polyethyleneimine-cellulose plates, and the phosphates separated by thin layer chromatography in 0.5 M ammonium formate, pH 4.3. Standard lanes containing 4 μl of 5 mM mono, di, and triphosphate were prepared to identify the nucleotides under UV light. The appropriate areas of the plates were cut out and counted by liquid scintillation. The entire lane was counted in sections containing the mono, di and triphoshate including intervening spaces. When calculating monphosphate phosphorylation the di and triphosphates were combined. In studies requiring DTNB and pHMB the dithiothreitol was omitted and the assay performed with 100 μM DTNB or pHMB. Competition studies were done with the above concentrations of dAMP and ddAMP with the addition of AMP or UMP to concentrations of 100, 200, 500, 1000 and 2000 μM in assays with cytosolic preparations and 50, 100, 200, 500, and 1000 μM in determinations using mitochondrial extracts.

Enzyme kinetics assays. Subcellular extracts from PBMCs or CEMss cells were incubated at 37 °C for 10 min for the determination of AMP phosphorylation parameters and 30 min using dAMP and ddAMP as substrates. A range of nucleotide monophosphate concentrations including 5, 10, 20, 50, 75, 100, 150, 200, 500, 750, and 1000 μM were used for these determinations. The resultant velocities were plotted, and the K_m and V_{max} values determined by Lineweaver-Burke plots. The protein concentrations were 0.1 μg for AMP and 1 μg for dAMP and ddAMP phosphorylation determinations with mitochondrial preparations and 1 μg for AMP and 10 μg for dAMP and ddAMP determinations with cytosolic fractions. The phosphorylation assays were performed as described above under "Nucleotide monophosphate assays".

TABLE 1. Phosphorylating Activity in Extracts of Mitochondria and Cytosol of Quiescent and Stimulated PBMC.

	Cytosol		Mitochondria	
Substrate	**pmol/min/μg**	**pmol/min/10^6 cells**	**pmol/min/μg**	**pmol/min/10^6 cells**
Q-PBMC:				
AMP	41 ± 2	116 ± 7	956 ± 99	295 ± 29
UMP	2.9 ± 0.20	1.2 ± 0.10	2.1 ± 0.10	0.9 ± 0.05
dAMP	1.5 ± 0.09	6.7 ± 0.5	1.6 ± 0.06	0.4 ± 0.02
ddAMP	1.3 ± 0.001	5.9 ± 0.01	2.1 ± 0.05	0.6 ± 0.01
PHA-PBMC:				
AMP	31.5 ± 2.7	581 ± 60	2405 ± 102	2966 ± 136
UMP	9.4 ± 1.2	61 ± 7	0.6 ± 0.03	1.2 ± 0.06
dAMP	1.5 ± 0.1	38 ± 1.8	6.9 ± 0.08	6.1 ± 0.07
ddAMP	1.4 ± 0.01	32 ± 0.2	6.2 ± 0.04	5.5 ± 0.02
CEMss:				
AMP	20 ± 6.5	928 ± 128	466 ± 94	2395 ± 216
UMP	7.9 ± 1.13	481 ± 72.3	0.55 ± 0.05	1.9 ± 0.64
dAMP	1.5 ± 0.04	78 ± 4.7	9.7 ± 0.3	67 ± 8.6
ddAMP	0.51 ± 0.08	27 ± 2.6	2.9 ± 0.09	20 ± 0.69

Extracts from stimulated and quiescent PBMCs were incubated with 50 μM [^{3}H] substrate and the unreacted substrate separated by TLC. The spots were cut out and counted by liquid scintillation to determine phosphorylation. Data are the means of three experiments.

RESULTS

Subcellular localization of adenylate kinase isozymes. Adenylate kinase activities were determined in subcellular fractions isolated from quiescent PBMC, PHA-stimulated PBMC and CEMss cells. As shown by the results in Table 1, AK activity in PBMCs and CEMss cells was present mainly in the mitochondria rather than cytosol. PHA stimulation of PBMC gave at least a 2-3 fold increase in the AK activity. A very different phosphorylation profile, however, was seen with ddAMP and dAMP. Thus, the mitochondrial fractions contained only about 15-17% of the total ddAMP and dAMP phosphorylating activity (on a cell basis) seen in these extracts of both resting and stimulated PBMCs. In contrast, phosphorylating activity for ddAMP and dAMP in CEMss cells was more evenly distributed between the mitochondria and cytosol than that in PBMC. UMP kinase activity was present mainly in the cytosol of both PBMC and CEMss cells.

Effect of sulfhydryl inhibitors. We examined the effect of the sulfhydryl reactive inhibitors, DTNB and pHMB, on the subcellular nucleotide phosphorylating activities seen in PBMCs [11,13,15,17,18]. These reagents will inhibit CMP-UMP kinase and AK1 activity but not AK2 activity. As shown in Table 2, both sulfhydryl compounds inhibited the cytosolic UMP

TABLE 2. Effect of DTNB and pHMB on ddAMP, AMP, and UMP Phosphorylation by the Mitochondria and Cytosol of Stimulated and Quiescent PBMC.

Substrate	Cytosol		Mitochondria	
	DTNB	pHMB	DTNB	pHMB
	% of control		% of control	
QPBMC:				
AMP	120 ± 4	104 ± 1	88 ± 2	93 ± 6
dAMP	131 ± 4	138 ± 3	117 ± 4	129 ± 1
ddAMP	118 ± 2	131 ± 1	154 ± 21	116 ± 2
UMP	11 ± 1	8 ± 1	77 ± 4	77 ± 2
PHA-PBMC:				
AMP	113 ± 4	104 ± 1	112 ± 5	91 ± 2
dAMP	93 ± 2	65 ± 6	96 ± 6	114 ± 12
ddAMP	86 ± 2	83 ± 1	106 ± 2	104 ± 3
UMP	18 ± 9	19 ± 6	106 ± 2	116 ± 4
CEMss:				
AMP	93 ± 7	105 ± 5	98 ± 2	98 ± 4
dAMP	74 ± 6	79 ± 6	97 ± 5	104 ± 2
ddAMP	86 ± 12	91 ± 2	98 ± 5	98 ± 6
UMP	0.5 ± 0.16	0.5 ± 0.15	ND	ND

Incubations for the determination of inhibition by sulfhydryl compounds were performed as described in "Materials and Methods" except dithiothreitol was omitted in these determinations. The phosphorylated products were separated from unreacted substrate by TLC and counted by liquid scintillation counting. Data are the means of three experiments.

kinase activity of both resting and PHA-stimulated PBMC, but had no significant effect on the AK activity seen in either the mitochondrial or cytosolic fraction indicating AK 2 activity. No inhibition of ddAMP phosphorylating activities by these agents was seen in either of the subcellular fractions. Only a slight inhibition (~15%) of the phosphorylating activity for dAMP was seen in the cytosol, but not mitochondria, of the PBMCs. We also performed experiments where various nucleotides were tested as competitors of these phosphorylations. Addition of AMP as might be expected, caused a complete inhibition of the phosphorylation of both ddAMP and dAMP in both mitochondrial and cytosolic enzymes. UMP exerted a partial inhibition (~40%) of phosphorylation of dAMP in the cytosol but no significant effect was noted on the phosphorylation of ddAMP in either the mitochondrial or cytosolic activities (Table 3).

Kinetic properties of mitochondrial and cytosolic kinase activities. The apparent K_m and V_{max} for ddAMP and dAMP phosphorylation were similar in value for the mitochondria and cytosolic enzyme activities of PBMCs. The efficiency of phosphorylation (V_{max}/K_m) of ddAMP and dAMP was only 1-2% that of AMP, indicating that these nucleotides are very poor substrates

TABLE 3. Inhibition of Phosphorylation by Natural Substrates of Adenylate Kinase and CMP/UMP Kinase

Substrate	Cytosol		Mitochondria	
	AMP	UMP	AMP	UMP
	% Inhibition		% Inhibition	
QPBMC:				
dAMP	92 ± 2	36 ± 2	86 ± 2	6 ± 6
ddAMP	84 ± 1	11 ± 1	82 ± 2	7.5 ± 7.5
PHA-PBMC:				
dAMP	94 ± 2	46 ± 4	90 ± 4	12 ± 5
ddAMP	82 ± 2	10 ± 1	92 ± 1	5 ± 4

Preparations from the indicated subcellular fraction from quiescent (Q-PBMC) and stimulated PBMC (PHA-PBMC) were incubated with 50 μM substrate and 1 mM of AMP or UMP.

for the kinases. It should be noted that no activity was detected with these two enzyme fractions when the reactions were carried out with GTP, instead of ATP, as the phosphate donor.

DISCUSSION

Adenylate kinase plays a well defined role in the homeostasis of adenine nucleotide metabolism, but its exact role in the conversion of pharmacological agents such as ddAMP has received limited attention. The present studies showed that the specific activity of adenylate kinase in human peripheral blood mononuclear cells is localized mainly in the mitochondria. These results are in agreement with previous studies that show in certain tissues such as liver, kidney and spleen adenylate kinase is localized primarily in the intermembrane space of the mitochondria [14]. However, in the case of ddAMP and the natural nucleotide dAMP, the major phosphorylating activity occurred in the cytosol rather than the mitochondria of these cells. Nave et al [10] previously showed ddAMP can be phosphorylated by AK1. However, the sulfhydryl inhibitors pHMB and DTNB, at concentrations known to inhibit AK1 activity, exerted no significant inhibitory effect on the subcellular kinase activities for AMP or ddAMP. These results, therefore, indicated that AK1 is not active in the PBMCs and that ddAMP phosphorylation in the cytosol of these cells involves an enzyme other than AK1. One possibility is that AK2 exists in the two different subcellular compartments or that there is leakage of the mitochondrial kinase into the cytosolic compartment during the isolation procedures. The latter seems unlikely since we did not detect any leakage of the mitochondrial marker enzymes, cytochrome c oxidase and glutamate dehydrogenase, in our cytosolic fractions. Thus these results support the concept that different enzymes, one being in the mitochondria and

TABLE 4. Kinetic Parameters of Phosphorylation in Mitochondrial and Cytosolic Extracts from Quiescent and PHA Stimulated PBMCs

	Acceptor					
	AMP	**2'-dAMP**	**2',3'-ddAMP**	**AMP**	**2'-dAMP**	**2',3'-ddAMP**
Parameter		**Cytosol**			**Mitochondria**	
Q-PBMC:						
K_m (mM)	0.11 ± 0.03	2.65 ± 0.11	1.82 ± 0.30	0.17 ± 0.01	1.77 ± 0.45	1.69 ± 0.15
V_{max} (pmol/min/μg)	256 ± 47	127 ± 12	121 ± 8	1014 ± 10	97 ± 11	128 ± 10
V_{max}/K_m	2327 ± 41	48 ± 4	67 ± 9	5884 ± 251	55 ± 12	76 ± 6
PHA-PBMC:						
K_m (mM)	0.12 ± 0.19	1.96 ± 0.22	1.55 ± 0.20	0.10 ± 0.05	1.36 ± 0.39	1.25 ± 0.50
V_{max} (pmol/min/μg)	267 ± 42	123 ± 14	101 ± 6	3075 ± 228	166 ± 10	151 ± 19
V_{max}/K_m	2225 ± 71	62 ± 14	65 ± 2	30637 ± 1062	122 ± 16	120 ± 24
CEMss:						
K_m (mM)	0.14 ± 0.02	1.19 ± 0.30	0.73 ± 0.06	0.19 ± 0.06	1.78 ± 0.40	0.53 ± 0.08
V_{max} (pmol/min/μg)	55 ± 1	74 ± 15	21 ± 1	2400 ± 68	165 ± 11	66 ± 7
V_{max}/K_m	392 ± 17	62 ± 12	28 ± 6	12631 ± 254	93 ± 6.9	128 ± 22

Cell extracts were incubated with a range of [^{3}H] substrate from 5-500 mM. Phosphorylation was measured by TLC and the kinetic parameters determined using Lineweaver-Burke analysis. Data are the means of three experiments ± the standard error.

another in the cytosol phosphorylate ddAMP and dAMP. The exact identity of the latter kinase activity remains to be identified, although it is possible that it represents an AK2 isozyme.

The rate of phosphorylation of ddAMP and dAMP by the different subcellular kinases was about 1-2% compared to that of AMP. This shows the very limited capacity of these kinases to catalyze the phosphorylation of nucleotides which lack both a 2' and 3'-hydroxyl group. However, it should be kept in mind that the level of adenylate kinase activity in lymphoid cells and probably macrophages is quite high, so that, even poor substrates can be effectively phosphorylated in these cells. PBMCs incubated with ddI at concentrations of 5 μM, sufficient to inhibit HIV replication, accumulated ddATP equivalent to a concentration of about 0.05 μM, several orders of magnitude lower than ATP concentrations in these cells, but apparently sufficient to inhibit HIV replication in whole cells. Thus, given the very low capacity of the cellular enzymes to anabolize the nucleotide analogs, the identification and characterization of the enzymes responsible for these phosphorylations are important in our understanding of the action of these drugs. Certain issues of interest arise from the present results. One is the potential role that mitochondrial AK2 plays in the peripheral neuropathy and pancreatitis sometimes seen during ddI treatment. These toxicities have generally been attributed to the metabolism of the drug by the mitochondria and consequent disruption of mitochondrial DNA metabolism [19,20]. Thus, our studies on the subcellular metabolism of ddI in human blood cells may help in our understanding of these cellular mechanisms of drug toxicity and provide a rational basis for drug design of new compounds.

ACKNOWLEDGMENTS

This work was supported in part by PHS grant RO1 AI27652, Cancer center (CORE) grant P30 CA21765 from the National Institutes of Health, and the American Lebanese Syrian Associated Charities.

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