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Metabolic Pathways for Activation of the Antiviral Agent 9-(2-Phosphonylmethoxyethyl)Adenine in Human Lymphoid Cells

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9-(2-Phosphonylmethoxyethyl)adenine (PMEA), the acyclic phosphonate analog of adenine monophosphate, is a promising antiviral drug with activity against herpesviruses, Epstein-Barr virus, and retroviruses, including the human immunodeficiency virus. In order to be active, it must be converted to the diphosphate derivative, the putative inhibitor of viral DNA polymerases. The metabolic pathway responsible for activation of PMEA is unclear. The metabolism of PMEA was investigated in human T-lymphoid cells (CEMss) and a PMEA-resistant subline ($CEMss^{r-1}$) with a partial deficiency in adenylate kinase activity. Experiments with [^{3}H]PMEA showed that extracts of CEMss phosphorylated PMEA to its mono- and diphosphate in the presence of ATP as the phosphate donor. No other nucleotides or 5-phosphoribosyl pyrophosphate displayed appreciable activity as a phosphate donor. Subcellular fractionation experiments showed that CEMss cells contained two nucleotide kinase activities, one in mitochondria and one in the cytosol, which phosphorylated PMEA. The PMEA-resistant CEMss mutant proved to have a deficiency in the mitochondrial adenylate kinase activity, indicating that this enzyme was important in the phosphorylation of PMEA. Other effective antiviral purine phosphonate derivatives of PMEA showed a profile of phosphorylating activity similar to that of PMEA. By comparison, phosphorylation of the pyrimidine analog (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine proceeded by an enzyme present in the cytosol. We conclude from these studies that adenylate kinase which has been localized in the intermembrane space of mitochondria is the major route for PMEA phosphorylation in CEMss cells but that another hitherto unidentified enzyme(s) present in the cytosol may contribute to the anabolism of the phosphonates.

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is a prototype of a new class of compounds, several of which are shown in Fig. 1. The acyclic nucleoside phosphonates demonstrate potent activity against a wide range of DNA and RNA viruses. PMEA is active against several herpesviruses, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) both in vitro and in vivo (7, 9, 10), Epstein-Barr virus (15), and several retroviruses, including the human immunodeficiency viruses (HIVs) (2–4, 8, 18). The in vivo antiviral efficacy of PMEA has been documented in various animal retroviral models of AIDS, including feline immunodeficiency virus infections in cats, murine leukemia/sarcoma virus infections in mice, and simian immunodeficiency virus in macaques (3–5, 9, 11, 16). PMEA has been shown to be particularly effective in preexposure prophylaxis of simian immunodeficiency virus infections in macaques and found to completely suppress viremia and disease symptoms in all the treated animals (28). PMEA is therefore of interest as a potential drug for the treatment of HIV infection and some of the opportunistic infections associated with AIDS. PMEA and its lipophilic prodrug bispivaloyloxymethyl(bispom)-PMEA have currently entered phase I/II clinical trials as treatment for HIV infections in AIDS patients (6).

The antiviral efficacy of PMEA is based on the capacity of its diphosphate derivative (PMEApp) to preferentially inhibit viral DNA replication with relative sparing of host DNA synthesis (2, 7, 8). Conversion of PMEA to this active metabolite is carried out by cellular enzymes; however, the exact pathway responsible for this activation to PMEApp remains unclear. We have recently reported (20) that the development of resistance to PMEA in the human lymphoid cell line CEMss is associated with an enhanced drug efflux and a reduced phosphorylation of PMEA to the diphosphate PMEApp. This latter effect is associated with a ~50% decrease in the activity of adenylate kinase (AK) (ATP:AMP phosphotransferase, EC 2.7.4.3) compared to that of the parental cells. These results implicate AK as a key enzyme in the activation of PMEA to its active form.

At least three different AK isoenzymes have been identified in mammalian cells (12, 14, 21, 24–26). These isoenzymes differ in amino acid sequence and tissue distribution (12, 24, 30). AK1 (originally called myokinase) exists in the cytosol and is the primary enzyme in skeletal muscle, brain, and erythrocytes, while AK2 is localized in the outer membrane spaces of mitochondria and is found primarily in liver, kidney, spleen, and heart (24). AK3, which has specificity for GTP as a phosphate donor, is more ubiquitous in tissues and is found in the mitochondrial matrix (24, 26).

The isoforms of AK and their subcellular distribution in lymphoid cells have not been determined. In this report, we have determined and compared the subcellular distribution of AK activities in PMEA-resistant and -sensitive human lymphoid cells (CEMss) and examined the specificity of the enzyme for PMEA and related phosphonate derivatives.

MATERIALS AND METHODS

Chemicals. All of the phosphonate analogs (for structures, see Fig. 1) PMEA, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) adenine (HPMPA), (*R*)-9-(2-phosphonylmethoxypropyl) adenine (PMPA), 9-(phosphonoylmethoxyethyl)-2,6-diaminopurine (PMEDAP), 9-(2,5-dihydro-5(phosphonomethoxy)-2-furanyl) adenine (D4AP), (*R*)-9-(2-phosphonylmethoxypropyl)-2,6-

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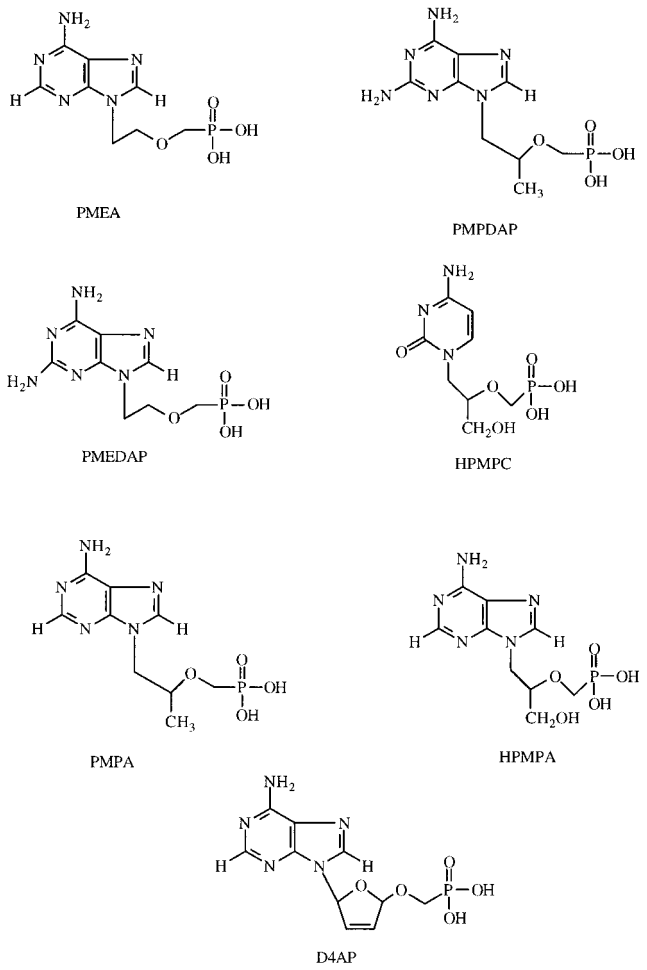

FIG. 1. The structural formulas of the phosphonates PMEA, PMEDAP, PMPA, PMPDAP, HPMPC, HPMPA, and D4AP.

diaminopurine (PMPDAP), and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) and the phosphorylated products of these compounds were kindly provided by Norbert Bischofberger, Gilead Sciences (Foster City, Calif.). The radioactive compounds PMEA [adenine-8-^{14}C], PMEDAP [adenine-8-^{3}H], PMPA [adenine-2,8-^{3}H], PMPDAP [adenine-8-^{3}H], HPMPA [adenine-2,8-^{3}H], HPMPC [cytidine-5-^{3}H], and D4AP [adenine-8-^{3}H] were obtained from Moravek Biochemicals (Brea, Calif.). Polyethyleneimine-cellulose plates were purchased from Baxter Scientific Products (McGraw Park, Ill.). 5,5′-dithio-bis(2-nitrobenzoate)(DTNB), *p*-hydroxy-mercuribenzoic acid (pHMB), pyruvate kinase, phosphoenol pyruvate (PEP), Trizma base, dithiothreitol (DTT), 5-phosphorylribose-1-pyrophosphate (PRPP), bovine serum albumin, and nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.). Media used for cell cultures were from Biowhittaker Inc. (Walkersville, Md.). Protease inhibitors were obtained as a kit from Boehringer Mannheim Corp. (Indianapolis, Ind.).

Cells and cell fractionation for the determination of AK distribution. CEMss cells were obtained from Peter Nara through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infections Diseases. The parental and PMEA-resistant CEMss$^{r\text{-}1}$ lines were grown as described previously (20). The resistant cells, when grown in the presence of PMEA, were subcultured into drug-free medium for at least 1 week before lysates were prepared. Cells in log phase were collected by centrifugation, washed twice with phosphate-buffered saline, and fractionated by the method of Richter (19). The cells were suspended at 2×10^8 cells per ml in MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 1 mM EDTA, pH 7.4) and homogenized in a glass homogenizer for 12 strokes. Protease inhibitors (1 mM Pefabloc, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM DTT) were added, and the cell lysate was centrifuged for 10 min at 800 × *g* at 4°C to sediment the nuclei. The resulting supernatant was centrifuged at 9,500 × *g* for 10 min at 4°C to sediment the mitochondria and at 105,000 × *g* to sediment the microsomal fraction. The mitochondrial pellet was washed twice with ice-cold MSH buffer and resuspended to 800 μl in MSH buffer plus protease inhibitors. The suspension was subjected to three freeze-thaw cycles to lyse the mitochondria and then stored in the presence of 10% glycerol and 3 mM DTT in liquid nitrogen until use. The cytosolic fractions were aliquoted and stored in liquid nitrogen until use. The microsomal pellets were suspended in 2 ml of MSH buffer and stored in liquid nitrogen until use. The protein concentrations of all fractions were determined prior to use with the Bio-Rad protein assay kit.

Nucleotide kinase assays and the compartment markers. AMP and UMP kinase activities were determined at 37°C in a reaction volume of 30 μl containing 0.05 M Tris buffer (pH 7.0), 3 mM DTT, 5 mM $MgCl_2$, 0.5 μg of bovine serum albumin per ml, 15 mM phospho(enol)pyruvate, pyruvate kinase (1 U) and 0.05 μg of the mitochondrial or 1.5 μg of the cytosolic fraction. The reaction was initiated with the appropriate radioactive substrate: [^{3}H]AMP (50 μM, 0.3 Ci/mol) or [^{3}H]UMP (50 μM, 0.3 Ci/mol). The reactions were stopped at 10 min by addition of 10 μl of 0.3 M EDTA, and 4-μl aliquots were immediately spotted on polyethyleneimine-cellulose plates, and the various phosphorylated products were separated by ascending thin-layer chromatography in 0.5 M ammonium formate, pH 4.3. Standard lanes (4 μl of 5 mM mono-, di-, and triphosphate derivatives) were prepared to permit identification of the nucleotide under UV light after chromatography. The nucleotide spots were cut out from the plates and counted by liquid scintillation counting.

In the assays for PMEA and other phosphonate derivatives the reactions were carried out in 0.1 ml (total volume) of the same components as above. Enzyme concentrations were 0.1 mg of the mitochondrial fraction or 1 mg of the cytosolic fraction. The reaction was initiated by addition of [^{14}C]PMEA (2 mM, 2.5 mCi/mol) or other phosphonate derivatives as indicated, and the mixture was incubated at 37°C for 2 h. The reactions were terminated by the addition of 0.25 ml of 98.5% ice-cold methanol at pH 7. The formation of PMEA monophosphate (PMEAp) and PMEApp were determined by anion-exchange high-pressure liquid chromatography (HPLC) using a series of linear gradients from 0.005 M (buffer A) to 0.6 M (buffer B) ammonium phosphate buffer. The gradient was 15 min from 0 to 30% buffer B, 10 min from 30 to 40% buffer B, and then 22 min at 100% buffer B with a flow rate of 1.5 ml/min.

TABLE 1. Subcellular distribution of enzyme activities in wild-type and PMEA-resistant CEMss$^{r\text{-}1}$ cells[a]

Substrate	Cell type	pmol/min/10^6 cells (% of cellular activity)		
		Mitochondria	Cytosol	Microsomes
AMP	CEMss	1,836 ± 155(71)	679 ± 267(26)	76 ± 10(4)
	CEMss$^{r\text{-}1}$	867 ± 437(62)	470 ± 115(34)	52 ± 11(4)
PMEA	CEMss	0.50 ± 0.18(56)	0.39 ± 0.074(44)	<0.1
	CEMss$^{r\text{-}1}$	0.26 ± 0.09(46)	0.30 ± 0.04(54)	<0.1
UMP	CEMss	2.1 ± 0.76(0.5)	356 ± 60.0(95)	12 ± 5.80(3.5)
	CEMss$^{r\text{-}1}$	1.7 ± 0.17(0.7)	255 ± 72(97)	6.2 ± 0.36(2.3)
Adenosine	CEMss	0.86 ± 0.23(1)	72.8 ± 15.83(99)	ND
	CEMss$^{r\text{-}1}$	0.47 ± 0.38(1)	71.9 ± 8.9(99)	ND
Cytochrome *c*	CEMss	990 ± 300(100)	<0.1	ND
	CEMss$^{r\text{-}1}$	1,310 ± 350(100)	<0.1	ND
Glutamate	CEMss	2,180 ± 620(100)	<0.1	ND
	CEMss$^{r\text{-}1}$	2,610 ± 420(100)	<0.1	ND

[a] Fractions were prepared and assayed as described in Materials and Methods. The data are the means of two experiments ± the range. ND, not determined.

TABLE 2. Inhibition of enzymatic activity by sulfhydryl reagents[a]

Cellular fraction	Substrate	% recovery of enzyme activity	
		DTNB (100 μM)	pHMB (100 μM)
Mitochondria	AMP	100 ± 5.0	90 ± 3.0
	PMEA	94 ± 4.0	88 ± 1.0
Cytosol	AMP	88 ± 6.0	96 ± 4.0
	PMEA	110 ± 8.0	120 ± 23
	UMP	<1	<1

[a] Fractions were prepared and assayed as described in Materials and Methods with the following modifications. Assay mixtures and controls were prepared by omitting DTT and adding the indicated concentration of DTNB or pHMB 15 min prior to the addition of label. The data are the means of two experiments ± the range.

Cytochrome *c* oxidase, a mitochondria marker of the outer membrane, was assayed essentially by the method of Storrie (23); oxidation of the reduced cytochrome *c* was measured at 550 nm, and the specific activity was calculated using an extinction coefficient of 19.6. Glutamate dehydrogenase, a mitochondrial matrix marker, was measured by the method of Schmidt (22) by following the NADH oxidation and using an extinction coefficient of 6.22. AK was measured by a radioactive assay essentially as described in earlier studies (29).

Data analysis. Kinetic parameters were calculated using a Lineweaver-Burk double reciprocal plot. Enzfitter, a program by Elsevier-Biosoft, was used to fit a line to the data by using weighted least squares linear regression.

RESULTS

PMEA phosphorylation activity of parental and resistant CEMss cells. We compared the effect of ATP and PRPP as phosphate donors on phosphorylation of PMEA in cell extracts of both the wild-type and PMEA-resistant CEMss^{r-1} cells. CEMss cell extracts incubated with 5 mM ATP and 2 mM PMEA formed 12.3 and 5.8 pmol of PMEAp and PMEApp, respectively, per min per mg of protein, while extracts of the resistant cells generated PMEAp and PMEApp half as efficiently as the wild type (data not shown). This decrease in PMEA phosphorylation paralleled the decrease in AMP kinase activity we have found in the CEMss variant (20). In contrast, there was no detectable phosphorylation of PMEA to PMEApp when PRPP was substituted for ATP as the phosphate donor. These results clearly demonstrate the role of AMP kinase in the phosphorylation of PMEA.

Intracellular distribution of AK activity. Cells were fractionated, and the subcellular localization of AK and PMEA phosphorylating activity(ies) was determined in wild-type and resistant CEMss^{r-1} cells. Approximately 70% of total AK activity in the CEMss cells was in the mitochondria, and 26 and 4% of the activity was in cytosolic and microsomal fractions, respectively (Table 1). Of note was that in the resistant cells the decrease in AK activity was mostly in the mitochondria. A similar profile of phosphorylating activity was observed with PMEA except that a somewhat higher (~40%) phosphorylating activity was found in the cytosol, as with AMP. HPLC analysis of the products also revealed that in the mitochondria PMEAp accumulated to a greater extent that PMEApp, at a ratio of 4:1, while in the cytosol both PMEAp and PMEApp were formed to about the same extent, albeit at a lower rate than in the mitochondria.

For comparison, we determined the subcellular expression of two other enzymes, UMP kinase and adenosine kinase. Essentially all the UMP kinase and adenosine kinase activity was associated with the cytosol or microsomal fractions (Table 1). These results indicated that the enzyme activities associated with the mitochondria in the CEMss cells are not likely to be due to an adherent cytoplasmic contamination. Cytochrome *c* oxidase and glutamate dehydrogenase, indicators of leakage of soluble enzymes from mitochondrial outer membrane space and matrix, respectively, were negligible (<0.1%) in the cytoplasmic supernatant (Table 1).

Effect of sulfhydryl reagents on nucleotide kinases. AK1 and UMP kinase are both highly susceptible to inactivation by sulfhydryl reagents such as pHMB and DTNB, whereas both AK2 and AK3 are insensitive to these inhibitors (12–14, 21). To determine possible differences between the mitochondrial and cytosolic enzyme activities, the effects of these two inhibitors on the phosphorylation of the various compounds by the subcellular fractions was studied. As shown in Table 2, the activity of UMP kinase was almost totally inhibited by DTNB and pHMB at a concentration of 100 μM, whereas essentially complete activity was retained for adenylate kinase and PMEA phosphorylation activities in both the mitochondrial and cytosolic fractions of these cells in the presence of these two inhibitors. In contrast, when AK1 (obtained commercially) was tested, complete enzyme inhibition was seen under the same conditions with these two sulfhydryl inhibitors. These results indicated that the phosphorylation of PMEA and AMP in both the mitochondria and cytosol of CEMss cells was not due to either AK1 or UMP kinase.

Comparison of the substrate specificity of mitochondrial and cytosolic enzymes. The phosphate donor specificity of various nucleoside triphosphates for the phosphorylation of the various substrates in the mitochondria and cytosol was examined. As shown in Table 3, ATP and dATP both acted as phosphate donors for adenylate kinase and UMP kinase, but only ATP acted as a phosphate donor for PMEA phosphorylation for both the mitochondrial and cytosolic enzymes. Relatively little activity was obtained with any of the other triphosphates tested with either the cytosolic or mitochondrial enzyme activities. These results show that none of the phosphorylating activities represent AK3, since this enzyme has specificity for GTP as the phosphate donor.

Table 4 shows the kinetic properties of adenylate kinase with AMP and PMEA as the phosphate acceptors. The substrate specificity for PMEA for the mitochondrial adenylate kinase was ~73-fold lower than that for the natural substrate AMP and the V_{max} of PMEA phosphorylation for the mitochondrial enzyme was at least 7,000-fold lower than for AMP. No major differences were found in the specificity of the cytosolic enzyme, except that the specific activity for the latter is considerably lower than in the mitochondrial fraction.

Substrate activity of related acyclic phosphonate. A number of structural analogs of PMEA (Fig. 1) were tested as substrates for the mitochondrial and cytosolic enzymes. Table 5

TABLE 3. Phosphate donor specificity of nucleoside monophosphate kinase activities in mitochondria and cytosol

Acceptor-donor pair	Activity (% of ATP control)[a]	
	Mitochondria	Cytosol
AMP-dATP	48 ± 6.0	71 ± 3.0
AMP-UTP	4.2 ± 1.4	0.1 ± 0.03
AMP-CTP	3.8 ± 0.5	<0.1
AMP-GTP	4.0 ± 0.6	<0.1
PMEA-dATP	<0.1	<0.1
UMP-dATP	100 ± 2.0	73 ± 6.0
UMP-UTP	37 ± 4.0	6.5 ± 2.3
UMP-CTP	35 ± 2.0	6.9 ± 2.2
UMP-GTP	35 ± 1.0	6.9 ± 0.2

[a] Activities are expressed as the percentage of the activity of the individual fractions with 2 mM ATP as the phosphate donor. Values are the average of two experiments ± the range.

TABLE 4. Kinetics of AMP kinase in mitochondria and cytosol[a]

Subcellular fraction and substrate	K_m (mM)	V_{max} (pmol/min/μg of protein)	V_{max}/K_m efficiency
Mitochondria, AMP	0.19 ± 0.06	2,400 ± 780	12,631
Cytosol, AMP	0.14 ± 0.02	55 ± 6.4	393
Mitochondria, PMEA	14.0 ± 2.8	0.34 ± 0.06	0.025
Cytosol, PMEA	15.9 ± 2.0	0.04 ± 0.01	0.003

[a] Data are the means of two experiments ± the range. The kinetics of AMP were determined at enzyme concentrations of 0.05 μg and 1.5 μg for the mitochondrial and cytosolic fractions, respectively. PMEA kinetics were determined at enzyme concentrations of 0.1 and 1 mg of mitochondrial and cytosolic fractions, respectively.

shows that all acyclic phosphonate derivatives of adenine tested (i.e., PMEDAP, PMPDAP, PMPA, D4AP, and HPMPA) behaved as substrates for both the mitochondrial and cytosolic enzymes, but with some significant differences. The 3-hydroxy-2-phosphonylpropyl derivative (HPMPA) was the most active substrate for both the mitochondrial and cytosolic enzymes. The order of phosphorylating activity was HPMPA> PMPA>D4AP>PMEA>PMPDAP>PMEDAP. By contrast, the pyrimidine counterpart of HPMPA (designated HPMPC), was a substrate for the cytosolic enzyme but not for the mitochondrial enzyme. It is noteworthy that the phosphorylation of HPMPC in the cytosol, unlike PMEA, was susceptible to inactivation by the inhibitors DTNB and pHMB (data not shown).

DISCUSSION

Previous studies have shown that the antiviral activity of PMEA is not related to preferential activation by viral enzymes. Balzarini et al. (1, 2) and Nave et al. (17) studied the phosphorylation of PMEA and various other antiviral nucleotide analogs such as 2′,3′-dideoxyadenosine monophosphate and 9-β-arabinofuranosyladenine monophosphate and found that these compounds could be converted directly to their diphosphate derivatives by the reverse action of PRPP synthetase isolated from either *Escherichia coli* or rat by using PRPP as phosphate donor. They suggested that PRPP synthetase could be involved in the phosphorylation of these compounds in intact cells. Our results, however, showed that PRPP synthetase was not involved in the phosphorylation of PMEA in the human T-lymphocytic cell line CEMss. The evidence was derived from our demonstration that (i) PRPP did not substitute for ATP as the phosphate donor in the phosphorylation of PMEA in cell extracts and (ii) a PMEA-resistant subline of CEMss cells demonstrated both diminished adenylate kinase activity and decreased phosphorylation of PMEA in the intact cells. Also, the PMEA-resistant cell line CEMss^{r-1} was just as sensitive as the wild type to 5-amino-4-imidazole carboxamide riboside, a compound which is anabolized via PRPP synthetase (20), further indicating that this enzyme is not a primary route for PMEA phosphorylation.

Subcellular localization studies showed that the major phosphorylating activity for PMEA and AMP in CEMss cells was associated with the mitochondria. Moreover, in the resistant cells the deficiency in the adenylate kinase was associated with the mitochondrial isozyme, clearly establishing this enzyme as the anabolic route for PMEA in this human T-cell line. The K_m of AK2 for PMEA was about 74-fold higher than the K_m for the natural substrate AMP. Despite this low substrate activity, the amount of phosphorylation associated with AK2 can account for the level of PMEA nucleotide accumulated in cells after incubations with inhibitory concentrations of the drug. Thus, extrapolation to conditions of 10 μM PMEA (an antiviral concentration) results in the potential accumulation of 1.2 pmol/10^6 cells by 8 h of incubation with PMEA, which compares to the actual accumulation of 0.2 pmol/10^6 cells seen in the intact cells during this time period.

About 40% of the total amount of measurable PMEA phosphorylating activity was found in the cytosolic fraction of CEMss cells (Table 1). The identity of this enzyme was not associated with either AK1 or NMK, since the compounds DNTB and pHMB, two known inhibitors of these enzymes, did not affect this phosphorylation. A possible explanation is that this represents some leakage of AK2 from the mitochondria to the cytosol during the fractionation; however, we did not find any significant leakage of marker enzymes from the mitochondria. Whatever the exact identity of this enzyme, our results with the resistant cells indicate that the cytosolic activity is not the primary route for anabolic activation of PMEA. Nevertheless, both enzyme activities may contribute to the metabolism of the analog in intact cells, but additional studies are needed to elucidate this question.

AKs obviously occupy a central role in adenine nucleotide metabolism in cells; however, their expression in different tissues can vary considerably. Tissues such as liver, kidney, spleen, and heart contain mainly AK2 in the mitochondria, while the cytosolic isozyme AK1 is predominant in tissue such as muscle, brain, and erythrocytes (24). In the present studies, the major kinase for AMP and PMEA in the human T-lymphocytic cell line CEMss was found in the mitochondria. Moreover, none of the kinases of PMEA or AMP, found in the cytosol or mitochondria, were inhibited by the sulfhydryls DTNB or pHMB. It is concluded from these results that the

TABLE 5. Comparison of phosphorylating activities for phosphonate analogs in wild-type and CEMss^{r-1} cells[a]

Compound	fmol/10^6 cells (% of total)			
	Mitochondria		Cytosol	
	CEMss	CEMss^{r-1}	CEMss	CEMss^{r-1}
PMEA	814 ± 58(70)	138 ± 10(38)	353 ± 17(30)	223 ± 15(62)
PMEDAP	402 ± 34(43)	168 ± 3(35)	534 ± 37(57)	316 ± 41(65)
(*R*)PMPDAP	497 ± 49(34)	132 ± 24(15)	964 ± 304(66)	769 ± 76(85)
(*R*)PMPA	1,929 ± 362(62)	461 ± 71(35)	1,189 ± 240(38)	870 ± 103(65)
D4AP	1,435 ± 81(60)	293 ± 80(36)	937 ± 110(40)	518 ± 61(64)
(*S*)HPMPA	6,453 ± 1,925(86)	1,231 ± 89(80)	1,028 ± 30(14)	316 ± 72(20)
(*S*)HPMPC			1,140 ± 408(100)	1,720 ± 86(100)

[a] These analogs were assayed under essentially the same conditions as PMEA, described in Materials and Methods. Concentrations of 2 mM of the analogs were incubated at 37°C for 2 h and separated in the same manner as PMEA. Data are the means of two experiments ± the range.

CEMss cells contain little, if any, expression of AK1 and that AK2 is the major enzyme operating in the phosphorylation of adenosine monophosphate analogs. An important question that remains for future investigation is whether a similar profile of AK activity is found in other more therapeutically relevant cells such as in peripheral blood lymphocytes and monocytes/macrophages.

Several other phosphonate analogs which were previously shown (8, 9, 16) to be active antiviral agents were also studied as substrates for AK. The active enantiomer S-HPMPA (3-hydroxy-2-phosphonomethoxypropyl) adenine and PMPA (2-phosphonylmethoxypropyl) adenine were the two most efficient substrates of the analogs tested for AK2 and for the cytosolic enzyme activity. By contrast, the two 2,6-diaminopurine derivative of PME (PMEDAP) and PMP (PMPDAP) were significantly less efficient substrates than the former compounds for the mitochondrial kinase but were phosphorylated two- to threefold better than PMEA by the cytosol. Since both PMEDAP and PMPDAP possess an amino group in the no. 2 position, it appears that subtle changes in the purine ring affect the ability of these compounds to act as substrates for AK2. It is also interesting that S-HPMPA and PMPA are both more active than PMEA against herpesviruses and retroviruses, respectively (9, 27). Thus, it seems possible that the increased antiviral activity of these two drugs is at least partly related to their increased phosphorylation. Obviously, enzymes other than just kinases (i.e., DNA polymerases, reverse transcriptase) are important determinants of the action of the phosphonates. Nevertheless, the present studies have revealed the underlying enzymatic basis for the intracellular anabolism of these novel antiviral agents in human lymphoid cells. The results show that both the cytosol and mitochondria contain purine nucleotide kinases that can convert these analogs to their active metabolites. The exact contribution of these alternate pathways to the metabolism and action of the phosphonate analogs and purine nucleotide analogs in general remains to be elucidated.

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