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Identification of Enzymes Catalyzing Two-Step Phosphorylation of Cidofovir and the Effect of Cytomegalovirus Infection on Their Activities in Host Cells

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SUMMARY

Cidofovir [CDV; (*S*)-1-(3-hydroxy-2-phosphonomethoxyethyl)-cytosine] is an acyclic nucleotide analog with potent and selective *in vitro* and *in vivo* activities against a broad spectrum of herpesviruses and other DNA viruses. We studied the mechanism of enzymatic synthesis of CDV diphosphate, the putative antiviral metabolite of CDV. The phosphorylation is two-step process catalyzed by several enzymes. An enzymatic activity phosphorylating CDV to its monophosphate derivative was purified from human liver and identified as pyrimidine nucleoside monophosphate kinase (EC 2.7.4.14.). CDV (K_m = 2.10 ± 0.18 mM and V_{max} = 1.10 ± 0.05 μmol/min/mg) was found to be a substantially weaker substrate for purified enzyme than CMP, UMP, or dCMP. Pyrimidine nucleoside monophosphate kinase was used for preparative enzymatic synthesis of CDV monophosphate. Pyruvate kinase (EC 2.7.1.40), creatine kinase (EC 2.7.3.2), and nucleoside diphosphate kinase (EC 2.7.4.6) were found to catalyze CDV diphosphate synthesis from CDV monophosphate, whereas phosphoglycerate kinase (EC 2.7.2.3) and succinyl-CoA synthetase (EC 6.2.1.4) did not. Based on V_{max}/K_m (phosphorylation efficiency) values determined with enzymes purified from human sources, the most efficient phosphorylation of CDV monophosphate is catalyzed by pyruvate kinase. After infection of human lung fibroblasts with cytomegalovirus, the intracellular activities of pyrimidine nucleoside monophosphate kinase, pyruvate kinase, creatine kinase, and nucleoside diphosphate kinase increased 2-, 1.3-, 3-, and 5-fold, respectively. The metabolism of [^{3}H]CDV in mock- and cytomegalovirus-infected cells was examined. The intracellular levels of CDV monophosphate and CDV diphosphate increased ~20- and 8-fold, respectively, in cytomegalovirus-infected cells, presumably due to the stimulation of CDV uptake and higher activities of phosphorylating enzymes.

Phosphonomethoxyalkylpurines and pyrimidines are acyclic nucleotide analogs with potent and selective antiviral activities (1). One of the most advanced compounds in this group, CDV [(*S*)-1-(3-hydroxy-2-phosphonomethoxyethyl)cytosine], has potent *in vitro* and *in vivo* antiherpetic activities (2) and has been recently approved for the treatment of HCMV retinitis in patients with AIDS (3). CDV is also involved in several other clinical trials as a topical agent against papillomavirus and herpesvirus infections (4).

In cells, CDV is metabolized predominantly to its monophosphate and diphosphate (5, 6). An additional intracellular metabolite was also observed and identified as CDVp-choline (5). The antiviral activity of CDV is due to the active metabolite of CDV, CDVpp, which preferentially inhibits viral DNA polymerases and serves as an alternative substrate with respect to dCTP (7). Unlike acyclovir or ganciclovir (8), the transfer of only two phosphate groups to CDV is necessary for the formation of CDVpp, and this process is independent of viral enzymes (6). In addition, antiviral activity of CDV is retained against acyclovir- or ganciclovir-resistant herpesviruses bearing mutations in genes for the viral kinases that are responsible for the first phosphorylation of these antiviral agents (9). In clinical trials, resistance to CDV has yet to be seen (10), and resistance *in vitro* is caused by mutations in the viral DNA polymerase gene.

In the case of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)-adenine, a close analog of CDV, AMP(dAMP) kinase was found to catalyze both phosphorylation steps to its monophosphate and diphosphate (11). 5-Phosphoribosyl-1-pyrophosphate synthetase may also play a role in the phosphorylation of this antiviral agent (12). Also, 9-(*S*)-(3-hydroxy-2-phosphonomethoxy-propyl)guanine is anabolized by two-step phosphorylation. The first step is catalyzation by GMP kinase, and NDP kinase participates in the second step (13). Preliminary data were provided on the phosphorylation of CDV that suggest that partially purified PNMP kinase from rat liver can catalyze the formation of CDVp (5). No

ABBREVIATIONS: CDV, cidofovir; CDVp, cidofovir monophosphate; CDVpp, cidofovir diphosphate; HCMV, human cytomegalovirus; NDP, nucleoside diphosphate; PNMP, pyrimidine nucleoside monophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; FCS, fetal calf serum; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

information has been reported regarding the conversion of CDVp to CDVpp.

Because phosphorylation is one of the crucial steps in the antiviral activity of CDV, we studied the mechanism of intracellular formation of CDVpp. An enzyme responsible for synthesis of CDVp was purified, and three different cellular kinases that are capable of catalyzing the formation of CDVpp from CDVp were identified. The kinetic data for both phosphorylation steps are presented. Also, the influence of HCMV infection on CDV intracellular metabolism and on the activity of respective kinases was examined, and the significance for the selective antiviral effect of the drug is discussed.

Materials and Methods

Chemicals. [5-^{3}H]CDV, [5-^{3}H]CMP, [5-^{3}H]dCMP, and [5-^{3}H]UMP were purchased from Moravek Biochemicals (Brea, CA). [5-^{3}H]CDP was obtained from Amersham (Arlington Heights, IL). All other nucleotides, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, leupeptin, pepstatin A, and bestatin were purchased from Sigma Chemical (St. Louis, MO). Nonidet P-40 was obtained as a 10% solution from Pierce Chemical (Rockford, IL). Pefabloc SC was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Enzymes. Pyruvate kinase from rabbit muscle, creatine kinase from bovine heart, NDP kinase from human erythrocytes, phosphoglycerate kinase from rabbit muscle, and succinyl-CoA synthetase from porcine heart were purchased from Sigma. Brain type of human creatine kinase (BB) was from Calzyme (San Luis Obispo, CA). Pyruvate kinase type M_2 was purified from human kidney (IIAM, Keystone Skin Bank, Exton, PA) through two-step chromatography on carboxymethylcellulose (Sigma) and Affi-Gel blue gel (BioRad, Hercules, CA) according to the procedure described previously (14). Purified enzyme had a specific activity of 110 μmol/min/mg in terms of ADP phosphorylation at 37°, and in accordance with the literature (15), its activity was stimulated by fructose-1,6-diphosphate and inhibited by L-phenylalanine.

Cells and virus. MRC-5 human embryonic lung fibroblast cells (American Type Culture Collection, Rockville, MD) were grown at 37° in 5% CO_2 atmosphere. The growth medium was Eagle's minimum essential supplemented with 10% FCS, 10^5 units/liter penicillin, and 100 mg/liter streptomycin. For all experiments, no passage of the cells past the tenth was used. High-titer stock of HCMV (Towne strain, American Type Culture Collection) was prepared from MRC-5 cells infected at a 0.05 multiplicity of infection. Fully cytopathic cells (14–16 days after infection) were scraped into the medium, collected through centrifugation, resuspended in a small volume of fresh medium, and disrupted through sonication. The stock was clarified by centrifugation at 6000 × *g* and stored at −70° in medium with 10% FCS. Usually, the virus titer in the stock was $0.8–1 \times 10^7$ plaque-forming units/ml when evaluated in MRC-5 cells.

Purification of CDV phosphorylating enzyme. Enzymatic assays during purification were performed at 37° in the presence of 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM DTT, 2 mM ATP, 1 mg/ml BSA, enzyme, and either 1 mM [^{3}H]CDV (specific activity, 100 μCi/μmol) or 200 μM [^{3}H]CMP (specific activity, 10 μCi/μmol) in a total volume of 10 μl. After the addition of substrate and product carrier standards, the reaction was stopped by spotting of aliquot volume onto a PEI-cellulose thin layer chromatography plate (Macherey-Nagel, Duren, Germany). After separation in 0.8 M LiCl/0.8 M acetic acid, the product spots were visualized under UV light, cut out, and evaluated for radioactivity in liquid scintillation cocktail. One unit of the enzymatic activity is defined as the amount of enzyme catalyzing phosphorylation of 1 μmol of CDV or CMP per 1 min at 37°.

The enzyme that phosphorylates CDV was purified from 20 ml of human liver homogenate (IIAM, Keystone Skin Bank); all procedures were carried out at 0–4°. All buffers contained 1 mM $MgCl_2$, 10 mM DTT, and 20% ethylene glycol. CDV phosphorylating activity was precipitated from the homogenate at 50–85% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min, dissolved in 20 mM Tris·HCl, pH 7.5 (buffer A), dialyzed overnight against 2× 1 liter of buffer A, and loaded onto a 40-ml Affi-Gel blue gel column equilibrated in buffer A. The column was washed with 200 ml of buffer A and with 60 ml of buffer A containing 1 mM ATP. The enzyme was eluted with 60 ml of buffer A supplemented with 1 mM ATP and 2 mM CMP. Finally, the column was washed with 80 ml of 1.5 M NaCl in buffer A. Column fractions (4 ml) were assayed after overnight microdialysis of fraction aliquots. Active fractions were pooled, and nucleotides were removed through three repeated cycles of dilution and concentration (from 15 to 1 ml). The sample was diluted in each cycle with 20 mM bis-Tris buffer, pH 6.0 (buffer B) and concentrated through ultrafiltration using Centriprep-10 (Amicon, Beverly, MA). After the final concentration step, the sample was loaded onto a 2-ml phosphocellulose P11 Whatman (Fairfield, NJ) column equilibrated in buffer B. The column was washed with 15 ml of buffer B and with 15 ml of 10 mM potassium phosphate, pH 8.0 (buffer C). The enzyme was eluted with 12 ml of buffer C containing 2 mM ATP. Finally, the column was washed with 300 mM potassium phosphate, pH 8.0. Active fractions were pooled, transferred into buffer A through one cycle of dilution/concentration, and loaded onto a 1-ml Resource-Q column (Pharmacia, Piscataway, NJ) equilibrated in buffer A. The column was washed with 10 ml of buffer A, and the enzyme was eluted with a 25-ml linear concentration gradient of 0–400 mM NaCl in buffer A. Active fractions were pooled, concentrated, and stored in −70°.

Enzymatic synthesis of nucleotides. CDV phosphorylating enzyme, partially purified on the Affi-Gel blue gel column, was used for preparative synthesis of CDVp, [^{3}H]CDVp, and [^{3}H]dCDP. Reaction mixture for CDVp synthesis contained 20 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM DTT, 3 mM ATP, 1 mg/ml BSA, 0.07 unit/ml enzyme, and 30 mM CDV. The incubation was carried out in a volume of 1 ml at 37° for 15 hr. After 3 and 6 hr of incubation, an additional 3 μmol of ATP was added to the reaction mixture. More than 20% of CDV was converted to CDVp under these conditions. Synthesis of [^{3}H]CDVp proceeded in 200 μl of reaction mixture containing 1 mM ATP, 0.015 unit of enzyme, 200 μCi of [^{3}H]CDV, and the other components as mentioned above. The sample was incubated at 37° for 10 hr. [^{3}H]dCDP was synthesized under the same conditions but [^{3}H]dCMP was used as a substrate instead of [^{3}H]CDV and the reaction was carried out for only 20 min with 0.002 unit of enzyme. The phosphorylation of both labeled compounds proceeded with 90% conversion. All synthesized nucleotides were purified using two chromatography steps. After removal of proteins through ultrafiltration, each sample was loaded onto a 4-ml Biogel 1000-Q column (Melcor Technologies, Sunnyvale, CA) equilibrated in 50 mM triethylammonium bicarbonate, pH 7.6. The reaction mixture was separated with linear gradient 50–350 mM triethylammonium bicarbonate for 90 min at a flow rate of 1 ml/min. The peak corresponding to the desired nucleotide was collected and evaporated to dryness. Residue of elution buffer was removed through chromatography on a Vydac C18 column (The Separations Group, Hesperia, CA). Samples were eluted with water. Each desired peak was collected, and the eluate was concentrated under vacuum. All nucleotides were obtained in >97% chemical or radioactive purity when evaluated by ion exchange high performance liquid chromatography.

Enzyme kinetics. All assays were performed at 37° in a total volume of 25 μl. Kinetic experiments with CDV phosphorylating enzyme were carried out under the reaction conditions described above. Kinetic constants were determined for [^{3}H]CDV (specific activity, 100 μCi/μmol), [^{3}H]CMP, [^{3}H]UMP, and [^{3}H]dCMP (the last three at a specific activity of 10 μCi/μmol).

Reaction mixtures for each enzyme involved in phosphorylation of CDVp contained 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 1

mg/ml BSA, one of the substrates tested ([^{3}H]CDVp at specific activity of 100 μCi/μmol or [^{3}H]CDP or [^{3}H]dCDP at a specific activity of 10 μCi/μmol), an appropriate amount of enzyme, and other specific components as follows: 100 mM KCl and 5 mM phospho(enol)pyruvate for the pyruvate kinase assay; 5 mM creatine phosphate for the creatine kinase assay; 5 mM ATP for the NDP kinase assay; 8 mM sodium phosphate, pH 7.5, 5 mM NAD^+, 5 mM DL-glyceraldehyde-3-phosphate, and 0.8 unit of glyceraldehyde-3-phosphate dehydrogenase for the phosphoglycerate kinase assay; and 5 mM sodium phosphate, pH 7.5, and 5 mM succinyl Co-A for succinyl Co-A synthetase.

Reaction mixtures from both phosphorylation steps were analyzed using thin layer chromatography as mentioned above. Each reaction was analyzed at three different time points, and kinetic constants were determined from initial reaction rates using the KinetAsist software (Think Technologies, San Diego, CA) based on the algorithm described by Cleland (16).

Preparation of cellular extracts and evaluation of enzymatic activities. Confluent MRC-5 cells were either mock-infected or infected with HCMV at a multiplicity of infection of 3. After adsorption of virus for 2 hr at 37°, the cell culture was washed with fresh medium and incubated in growth medium supplemented with 2% FCS. Thirty-six hr later, cells were washed with HEPES-buffered saline solution, harvested by trypsinization, resuspended in trypsin-neutralizing solution (Clonetics, San Diego, CA), collected through centrifugation, and washed in HEPES-buffered saline solution. After centrifugation, the pellet of 2×10^7 cells was resuspended in 400 μl of 20 mM Tris·HCl, pH 7.5, containing 5 mM DTT, 10% glycerol, 0.2% Nonidet P-40, 0.2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, and protease inhibitors (0.3 mg/ml Pefabloc, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 μg/ml bestatin, and 0.1 mM EGTA) and solubilized on ice for 30 min. The crude cell lysate was clarified through centrifugation at 50,000 × *g* for 30 min at 4°. The supernatant was desalted on a HiTrap desalting column (Pharmacia). Finally, the extract was concentrated to 200 μl using Microcon-10 (Amicon) and stored in aliquots at −70° until use. The activity of kinases was determined under the conditions described above. CDV and CDVp were present at 200 and 500 μM concentrations, respectively, and 5 mM phosphate donors were used.

Intracellular metabolism of CDV. Confluent MRC-5 cells in 75-cm^2 flasks were either mock-infected or infected with HCMV under the conditions described above. [^{3}H]CDV (specific activity, 1 Ci/mmol) at 1 μM concentration was added 12 hr after infection. Cells were incubated in the presence of the drug for 24, 48, or 72 hr and harvested as described above. Cell pellets were extracted twice with ice-cold 60% methanol according to published procedure (17). An aliquot of each extract was separated on 20-cm PEI-cellulose plates in 0.4 M LiCl (R_F: CDVpp, 0.06; CDVp, 0.18; CDV, 0.44; CDVp-choline, 0.92). After chromatography, the plates were cut into 1-cm slices, and the intracellular level of CDV metabolites was determined after evaluation of each slice for radioactivity.

Results

Purification and characterization of the CDV phosphorylating enzyme. Enzymatic activity capable of catalyzing the phosphorylation of CDV has been purified from human liver homogenate using a four-step purification procedure (Table 1). Fractions from each column were assayed for phosphorylation of CDV or CMP, and in all purification steps both activities copurified (Fig. 1). In the liver homogenate and after ammonium sulfate precipitation, we found phosphorylation to CDVpp or CTP, respectively, in the presence of ATP. However, after all subsequent purification steps, the phosphorylation products that were detected were CDVp or CDP, respectively. The ratio between CDV and CMP phosphorylating activities remained constant during all three column purification steps (CDV phosphorylation/CMP phosphorylation = 0.0045–0.0055). It was lower in earlier stages of the purification (CDV phosphorylation/CMP phosphorylation = 0.0025), probably due to the presence of interfering activities. By following this procedure, we were able to purify the CDV phosphorylating activity to near-homogeneity. Specific activity of the purified enzyme increased >3600-fold in terms of the CDV phosphorylation in comparison with crude liver extract. Silver stained sodium dodecyl sulfate-polyacrylamide gel after electrophoresis of purified enzyme revealed one dominant band with a molecular mass of 27 kDa. After gradient polyacrylamide gel electrophoresis under nondenaturing conditions, the CDV and CMP phosphorylating activities were found to comigrate with the stained protein (Fig. 2). These data suggest that the enzyme catalyzing phosphorylation of CDV to its monophosphate derivative is PNMP kinase. The molecular mass of human PNMP kinase was previously shown to be 28 kDa (18), which corresponds well with the value found for CDV phosphorylating enzyme. Also, the conditions under which this enzyme was eluted from both Affi-Gel blue gel and phosphocellulose columns were similar to those described for PNMP kinase (19).

The kinetic constants for phosphorylation of CDV by the purified kinase were compared with values for CMP, UMP, and dCMP. These nucleotides were shown to be preferred natural substrates for PNMP kinases from different mammalian sources, including humans (18). The K_m value for CDV was 2.10 ± 0.18 mM, and the phosphorylation proceeded with a V_{max} value of 1.10 ± 0.05 μmol/min/mg. The efficiency of phosphorylation (V_{max}/K_m) of natural substrates was found to be, in decreasing order, CMP > UMP > dCMP

TABLE 1

Summary of purification of the CDV phosphorylating activity.

Enzymatic activities were evaluated after each purification step in pooled fractions at 3 mM and 200 μM concentration of CDV and CMP, respectively. The assays were carried out under conditions described in Materials and Methods.

Purification step	Volume	Total activity		Proteins	Spec. activity[a]	Recovery[a]	Fold Purification[a]
		CDV	CMP				
	ml	*units*		*mg*	*10^{-3} units/mg*	*%*	
Human liver homogenate	20	0.112	42.0	387.6	0.29	100	1
$(NH_4)_2SO_4$	15	0.095	37.8	123.2	0.77	88	2.7
Affi-Gel blue gel column	30	0.088	19.3	1.28	69	81.5	238
Phosphocellulose column	3	0.049	8.8	0.72	680	45.3	2,345
Resource Q column	1.5	0.019	4.1	0.018	1,060	17.6	3,655

[a] In terms of CDV phosphorylating activity.

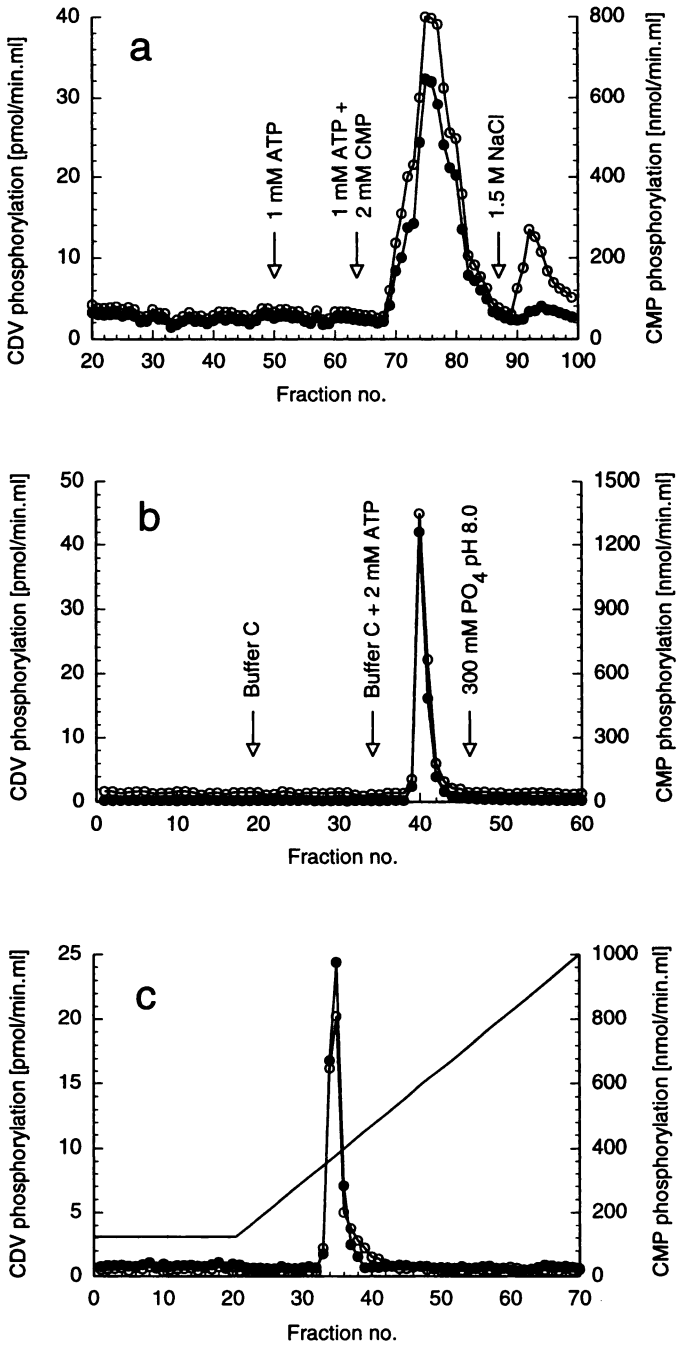

Fig. 1. Purification of CDV phosphorylating activity. a, Affi-Gel blue gel column. b, Phosphocellulose column. c, Resource-Q column. ●, CDV phosphorylating activity; ○, CMP phosphorylating activity.

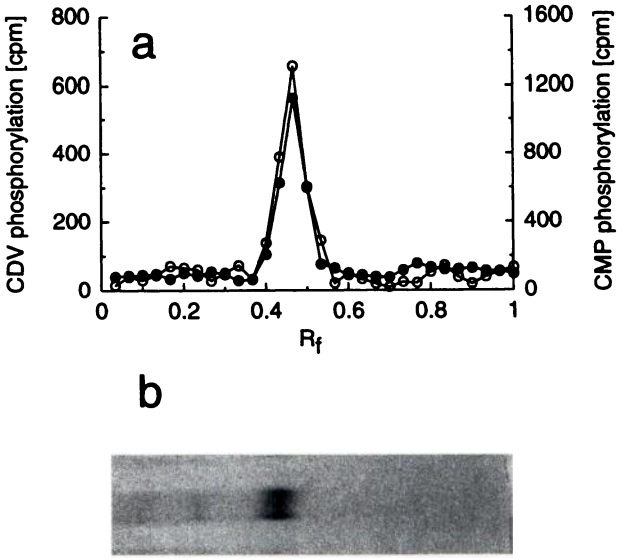

Fig. 2. Native polyacrylamide gel electrophoresis of CDV phosphorylating enzyme; 2 × 50 ng of the purified enzyme was loaded onto a 4–20% gradient polyacrylamide gel (BioRad) that had been pre-electrophoresed for 3 hr in Tris/glycine buffer containing 10 mM DTT. After electrophoretic separation (4 hr, 10 V/cm at 4°), one part of the gel was stained with silver, and the other was cut into 2-mm slices. CDV (●) and CMP (○) phosphorylating activities were determined after extraction of each slice. a, Enzyme activities. b, Silver-stained portion of the gel.

TABLE 2

Kinetic constants of phosphorylation reactions catalyzed by CDV phosphorylating enzyme

The enzyme purified from human liver was used for determination of kinetic constants of different phosphorylation reactions. All reactions were carried out at 37° and reaction mixtures were separated using thin layer chromatography. After determination of initial reaction rates the kinetic constants with standard errors were calculated from double-reciprocal plots using KinetAsist computer software.

Substrate	K_m	V_{max}	V_{max}/K_m
	μM	μmol/min/mg	
CDV	2,100 ± 180	1.10 ± 0.05	9.02×10^{-4}
CMP	8.0 ± 1.6	484 ± 68	60.5
dCMP	340 ± 9	107 ± 2	0.315
UMP	52.0 ± 4.0	996 ± 52	19.15

(Table 2), which is characteristic for PNMP kinase (20). CDV phosphorylation proceeded with three to five orders of magnitude lower efficiency compared with the natural substrates.

Enzymes catalyzing phosphorylation of CDVp. The phosphorylation of nucleoside diphosphates to their corresponding triphosphates can be catalyzed by several enzymes. The key enzyme for this step of nucleotide metabolism is NDP kinase (21), but several enzymes in the glycolytic pathway and citric acid cycle can also play a role in the formation and regulation of the intracellular level of nucleoside triphosphates (22, 23). Five enzymes from mammalian sources (pyruvate kinase, creatine kinase, NDP kinase, phosphoglycerate kinase, and succinyl-CoA synthetase) that are known to be able to transfer the phosphate group from different types of donor molecules to nucleoside diphosphates were tested for their ability to phosphorylate CDVp, CDP, and dCDP. Table 3 shows that all five enzymes are able to catalyze both CDP and dCDP phosphorylation. However, only pyruvate kinase, creatine kinase, and NDP kinase can utilize CDVp as a substrate. We pursued this finding in greater detail and evaluated the kinetics of CDVp phosphorylation by these three enzymes purified from human sources. For comparison, the kinetic constants for CDP and dCDP phosphorylation were also measured. The K_m values of CDVp are in the low millimolar range for all of these enzymes. The V_{max} value was found to be very similar for CDVp phosphorylation catalyzed by NDP kinase and pyruvate kinase and ~5-fold lower for the creatine kinase catalyzed reaction (Table 4). For the natural nucleotides, all three enzymes preferred CDP to dCDP, which is in agreement with previous findings (22–24). A comparison of phosphorylation efficacy (V_{max}/K_m) indicated a substantial difference between CDVp and the natural

TABLE 3

Phosphorylation of CDV monophosphate and NDPs by different mammalian kinases

Enzymatic reactions were performed under conditions described in Materials and Methods. All three substrates were present at 10 μM concentration and all phosphate donors at 5 mM concentration. Reaction mixtures were incubated at 37°, separated using thin layer chromatography and the initial reaction rates were determined.

Enzyme (Source)	Predominant reaction catalyzed	Rate of reaction		
		CDP	dCDP	CDVp
		nmol/min/mg		
Pyruvate kinase (Rabbit muscle) EC 2.7.1.40	ADP + phospho(enol)pyruvate = ATP + pyruvate	150	6.0	0.021
Creatine kinase (Bovine heart) EC 2.7.3.2	ADP + creatine phosphate = ATP + creatine	1.5	0.65	0.0052
NDP kinase (Human erythrocytes) EC 2.7.4.6	NDP + ATP = NTP + ADP	1,340	248	0.064
Phosphoglycerate kinase (Rabbit muscles) EC 2.7.2.3	ADP + 1,3-diphospho-D-glycerate = ATP + 3-phospho-D-glycerate	45.0	1.31	0
Succinyl-CoA synthetase (Porcine heart) EC 6.2.1.4	GDP + P_i + succinyl Co-A = GTP + succinate + Co-A	3.4	0.98	0

TABLE 4

Kinetics of phosphorylation of CDVp and natural NDPs by three different human kinases

Enzymes purified from human tissues were used for estimation of kinetic constants of different phosphorylation reactions. All reactions were carried out at 37° and reaction mixtures were separated using thin layer chromatography. The kinetic constants with standard errors were calculated from initial reaction rates using KinetAsist computer software.

Enzyme	Substrate	K_m	V_{max}	V_{max}/K_m
		mM	*μmol/min/mg*	
Pyruvate kinase	CDVp	5.80 ± 0.89	0.021 ± 0.002	3.6×10^{-3}
	CDP	4.36 ± 0.45	20.3 ± 1.3	4.6
	dCDP	4.76 ± 1.42	2.53 ± 0.45	0.52
Creatine kinase	CDVp	7.77 ± 0.83	0.0040 ± 0.0004	5.1×10^{-4}
	CDP	3.88 ± 0.44	0.174 ± 0.016	4.5×10^{-2}
	dCDP	3.44 ± 0.90	0.034 ± 0.005	9.7×10^{-3}
NDP kinase	CDVp	6.57 ± 0.91	0.0192 ± 0.002	2.9×10^{-3}
	CDP	0.70 ± 0.07	63.0 ± 4.5	90.0
	dCDP	2.47 ± 0.24	53.1 ± 4.3	21.5

cytosine nucleotides, especially for NDP kinase and pyruvate kinase. For creatine kinase, the difference in efficacy was less pronounced. Creatine kinase phosphorylates CDVp with an efficiency of 1.1% and 5.2% of that for CDP and dCDP, respectively (Table 4).

Effect of HCMV infection on the intracellular levels of enzymes phosphorylating CDV and CDVp. The activities of PNMP kinase, pyruvate kinase, creatine kinase, and NDP kinase were measured in cellular extracts prepared from mock-infected and HCMV-infected MRC-5 cells. Because some of these enzymes are present in cells in both soluble and membrane-bound form (25) or in subcellular compartments (26), a mixture of nondenaturing detergents for the disruption of cells was used. CDV and CDVp were incubated with cellular extracts prepared 36 hr after HCMV infection. Corresponding phosphate donors were present at 5 mM concentrations, which is ≥3-fold higher than their K_m values (15, 23, 24). All four enzymes were easily detectable in extracts prepared from mock-infected and HCMV-infected cells (Fig. 3). HCMV-infected cells contained higher activity for all kinases investigated than did the mock-infected cells. Phosphorylation of CDV in the presence of ATP proceeded twice as fast in extract prepared from HCMV-infected cells, suggesting higher activity of PNMP kinase. Also, we found substantially higher phosphorylation of CDVp catalyzed by NDP kinase and creatine kinase, with ~5- and ~3-fold increases in the HCMV-infected cells, respectively. Pyruvate kinase, the most active enzyme in terms of CDVp phosphorylation in uninfected cells, was only marginally increased (~1.3-fold) in cells infected with HCMV. We also determined the activities of phosphoglycerate kinase and succinyl-CoA synthetase, which were shown to be unable to catalyze *in vitro* CDVp phosphorylation. Phosphoglycerate kinase exhibited only very low activity (~10% of that of NDP kinase in uninfected cells), and HCMV infection had no measurable effect on its activity. No CDVp phosphorylation was detected in either type of cell extract when examined for succinyl-CoA synthetase activity (data not shown).

Recently, pyrophosphorylation of several other nucleoside phosphonates catalyzed by 5-phosphoribosyl-1-pyrophosphate synthetase purified from human erythrocytes has been reported (12). Therefore, we also investigated this possible mechanism for CDV activation. However, in neither the extract from mock-infected nor that from virus-infected cells was any pyrophosphorylation of CDV in the presence of 5-phosphoribosyl-1-pyrophosphate detected.

Effect of HCMV infection on intracellular metabolism of CDV. To study the effect of HCMV infection on CDV intracellular phosphorylation, the metabolism of 1 μM CDV was compared in mock-infected and HCMV-infected MRC-5 cells. An analysis of cellular extracts after incubation with the drug for 24, 48, and 72 hr revealed a substantial increase in the CDV concentration in infected cells. Although the total intracellular level of all CDV metabolites in mock-infected cells increased slowly during the time of incubation, their concentration in virus-infected cells reached peak levels between 24 and 48 hr after the addition of CDV. At that point, the total concentration of drug metabolites in infected cells was ~10-fold higher compared with mock-infected cells (Fig. 4). All four CDV metabolites (CDV, CDVp, CDVpp, and CDVp-choline) were detectable in mock-infected and HCMV-infected cells. A substantial elevation of each metabolite was

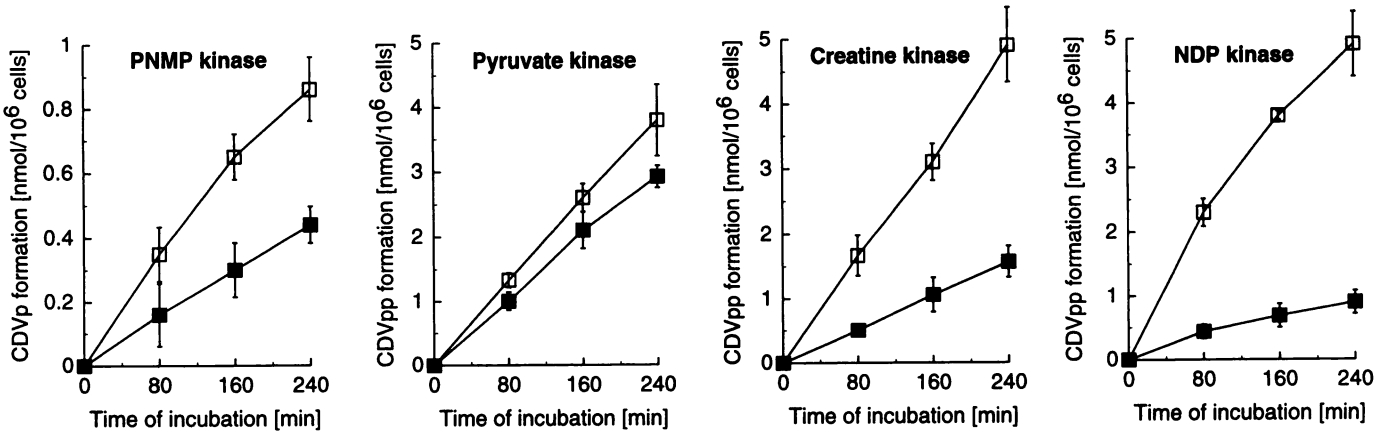

Fig. 3. Effect of HCMV infection on the intracellular activity of enzymes participating in CDV phosphorylation. MRC-5 cells were either (■) mock-infected or (□) infected with HCMV. Cells were harvested after 36 hr and extracted, and the activities of PNMP kinase, pyruvate kinase, creatine kinase, and NDP kinase were determined in cellular extracts as described in Materials and Methods. Data represent mean ± standard error of two independent experiments.

found in the virus-infected cells. After 24 hr in the presence of the drug, the intracellular concentrations of CDVp and CDVpp in the HCMV-infected cells were 0.97 and 0.71 pmol/10^6 cells, respectively (Table 5). These values are 20-fold higher for CDVp and 8-fold higher for CDVpp than those obtained from mock-infected cells. Also, the level of CDVp-choline was elevated in cells after HCMV infection. In the later phase of incubation with the drug, the concentration of CDV metabolites started to drop in the infected cells due to the virus cytopathic effect.

Discussion

CDV belongs to a group of highly effective antiviral agents known as acyclic nucleoside phosphonates. Although the phosphorylation pathways of some nucleoside phosphonates have been elucidated, the enzymes catalyzing the phosphorylation of CDV have not been identified. The CDV phosphorylating enzyme was purified from human liver extract and was identified as PNMP kinase on the basis of its properties during purification, molecular weight, and substrate specificity. Kinetic data obtained with the purified enzyme indicate that phosphorylation of CDV is three to five orders of magnitude less efficient than phosphorylation of CMP, UMP, and dCMP.

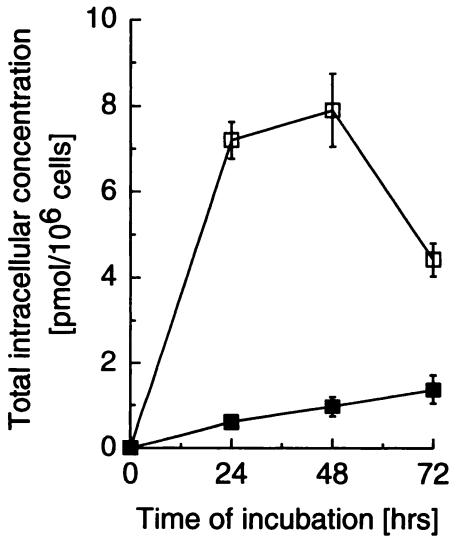

Fig. 4. Comparison of total intracellular concentration of CDV metabolites in (■ mock-infected and (□) HCMV-infected human lung fibroblasts. [^{3}H]CDV (1 μM) was added into the medium at 12 hr after infection, and the cells were incubated for the indicated times in the presence of the drug. After harvesting of the cells, radioactivity was evaluated in cellular extracts, and the intracellular level of the drug was calculated. Data represent mean ± standard error of two independent experiments.

Nucleoside diphosphates are substrates for several cellular kinases that have broader substrate specificity than enzymes that phosphorylate corresponding monophosphates (22–24). This fact suggests that several enzymes may catalyze the second step of CDV phosphorylation. Five mammalian kinases that phosphorylate nucleoside diphosphates in the presence of respective phosphate donors were studied for their ability to phosphorylate CDVp. Under physiological conditions, all of the five enzymes catalyze reactions that either proceed in the direction of nucleoside triphosphate formation [pyruvate kinase (27), phosphoglycerate kinase (28), and succinyl-CoA synthetase (29)] or are freely reversible [creatine kinase (30), NDP kinase (21)]. Although all five kinases tested phosphorylate both CDP and dCDP, only pyruvate kinase, creatine kinase, and NDP kinase were found to be able to catalyze the formation of CDVpp. The kinetics of CDVp, CDP, and dCDP phosphorylation were studied with these three enzymes purified from human sources. Both pyruvate kinase (15) and creatine kinase (30) exist in the form of at least four isoenzymes that differ in their molecular and kinetic characteristics. In this study, the M_2 type of pyruvate kinase and the BB type of creatine kinase were used. These forms are predominantly expressed in tissues hosting CDV-susceptible viruses. Kinetic data showed that pyruvate kinase is the most efficient enzyme for the *in vitro* phosphorylation of CDVp.

One of the important aspects for CDVpp formation is the intracellular level of the phosphorylating enzymes. Activities of all four kinases involved in CDV phosphorylation pathway were measured in extracts from human embryonic lung fibroblast. In these cells, a high level of phosphorylated forms of CDV was previously detected (5, 6). The highest CDVp phosphorylating activity found in cell extract is pyruvate kinase [0.75 nM/hr/10^6 cells], which corresponds to the data

TABLE 5

Effect of HCMV infection on intracellular concentration of CDV metabolites

MRC-5 cells were infected with HCMV and 12 hours later 1 μM [^{3}H]CDV was added into culture medium. Cells were incubated for indicated time in the presence of the drug, then harvested and extracted. Aliquots of the extracts were separated by thin layer chromatography and intracellular levels of the drug and its metabolites were determined. The values shown represent mean ± standard error from two independent experiments.

Time	Infection	Intracellular concentration				
		Total	CDV	CDVp	CDVpp	CDVp-choline
				pmol/10^6 cells		
24 hr	−	0.60 ± 0.14	0.29 ± 0.07	0.05 ± 0.02	0.09 ± 0.02	0.17 ± 0.06
	+	7.22 ± 0.42	5.03 ± 1.01	0.97 ± 0.21	0.72 ± 0.14	0.50 ± 0.11
48 hr	−	0.98 ± 0.24	0.39 ± 0.05	0.07 ± 0.02	0.19 ± 0.05	0.33 ± 0.02
	+	7.90 ± 0.84	4.47 ± 0.68	1.47 ± 0.17	1.10 ± 0.19	0.86 ± 0.19
72 hr	−	1.39 ± 0.33	0.58 ± 0.11	0.09 ± 0.03	0.29 ± 0.06	0.44 ± 0.07
	+	4.39 ± 0.38	2.39 ± 0.46	0.59 ± 0.13	0.56 ± 0.18	0.85 ± 0.22

obtained from *in vitro* experiments. Creatine kinase and NDP kinase were also shown to have activity but only ~50% and ~20% of that of pyruvate kinase, respectively. PNMP kinase had an activity of 0.14 nM/hr/10^6 cells in terms of CDV phosphorylation. These results are in accordance with findings that the least abundant intracellular metabolite of CDV is its monophosphate (5, 6). Therefore, the formation of CDVp probably is the rate-limiting step in the phosphorylation pathway. This situation changes dramatically in cells infected with HCMV, however. Although only a negligible increase in pyruvate kinase activity was detected in HCMV-infected cells, the levels of PNMP kinase and creatine kinase increased ~2- and ~3-fold, respectively, and the activity of NDP kinase increased 5-fold when evaluated 36 hr after HCMV infection. Increasing activity of creatine kinase correlates with the previous finding that HCMV immediate-early gene products can *trans*-activate expression of the cellular gene that encodes the BB creatine kinase. A >2-fold higher level of mRNA has been found in human fibroblasts 24 hr after infection, with a continual increase during the course of infection (31, 32). However, to date this finding has not been demonstrated at the level of enzymatic activity. Increased levels of PNMP kinase and NDP kinase have not been reported. It is known that HCMV possesses proven oncogenic potential and like other tumor viruses stimulates host cell macromolecular synthesis in infected cells (33–35). Recently, stimulation of the expression of several cellular proteins involved in host DNA synthesis, including DNA polymerase α (36) and thymidine kinase (31), has been shown. Also, no characteristic sequences suggesting genes coding for PNMP kinase or NDP kinase have been identified in the HCMV genome (37). Based on these data, we assume that stimulation of the expression of cellular enzymes during HCMV infection could account for the elevated activity of these two enzymes.

To characterize the influence of elevated activity of kinases on CDV intracellular phosphorylation, we compared the metabolism of the drug in MRC-5 cells that were either mock-infected or infected with HCMV. Both stimulation of cellular uptake of CDV and elevation of the intracellular levels of CDV monophosphate and diphosphate were found in virus-infected cells. The mechanism of stimulation of cellular uptake of CDV during HCMV infection is probably directly connected with the ability of this virus to stimulate expression of many different cellular genes (as discussed above) and, consequently, some transport processes in infected cells (38, 39). This influence on host cells is different for HCMV than for herpes simplex virus type 1 because no changes were found in CDV uptake and metabolism (5) or the in activity of the respective kinases (8) after infection with the latter virus.

In mock-infected cells, the same slow kinetics of accumulation were observed for both parental drug and its phosphorylated forms. The percentage conversion of CDV to CDVpp, calculated as the sum of the intracellular concentrations of CDVpp and CDVp-choline, remains approximately constant (43–53%) throughout the 72-hr incubation period, which suggests that the transport of the drug across plasma membrane is the primary limiting step determining the intracellular level of CDVpp. It has recently been shown that fluid-phase endocytosis is the likely mechanism of CDV uptake into cells (40). This nonspecific process is much less efficient than active receptor-mediated forms of cellular transport and is consistent with the observed slow continual accumulation of the drug. As mentioned above, the intracellular conversion of CDV to CDVpp was found to be relatively high despite the low catalytic efficiency of both phosphorylation steps. This may be explained in part by the slow limiting uptake of CDV and in part by the high efficiency of phosphorylation of natural nucleoside monophosphates and diphosphates, resulting in a fast turnover and a low intracellular concentration of these potentially competing molecules. The apparent stability of CDVpp, which can be assumed from its long intracellular half-life (5, 6), may also play an important role in the accumulation of this metabolite inside the cells. Long intracellular persistency of CDVpp is one of the major issues that enables infrequent dosing of CDV during the treatment of HCMV infections (3).

Previously, choline phosphate cytidylyltranferase (EC 2.7.7.15) was shown to catalyze the formation of CDVp-choline from CDVpp (5). When combined with data from the current study, we can present the scheme of CDV intracellular anabolism (Fig. 5). Enzymes that have been identified as participating in the phosphorylation of CDVp are expressed in most types of tissues, but their levels vary according to the tissue origin (21, 30). Also, the intracellular concentrations of nucleoside diphosphates that compete with CDVp differ according to the type, metabolic state, and proliferating activity of the cells. Thus, no general conclusion can be drawn about which of the three kinases plays a key role in the phosphorylation of CDVp. In slowly proliferating tissues, pyruvate kinase and creatine kinase would probably synthesize most of the CDVpp because NDP kinase expression was shown to be proliferation dependent (41) and this enzyme has higher affinity for natural nucleoside diphos-

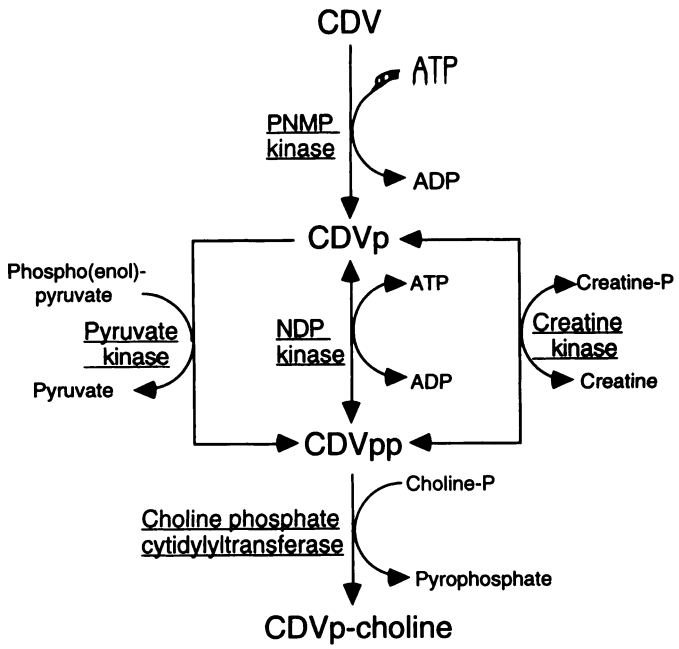

Fig. 5. Intracellular anabolism of CDV.

phates. However, infection with HCMV can change the contribution of each enzyme to CDVpp formation.

The results of the current study suggest that one of the aspects allowing CDV to act as an extremely potent inhibitor of productive HCMV infection is higher intracellular concentration of CDVpp induced in HCMV-infected cells. On the other hand, CDV can be effective as a prophylactic agent because the activity of respective kinases ensures its phosphorylation in uninfected cells.

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