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Phosphorylation of 2′,3′-Dideoxyinosine by Cytosolic 5′-Nucleotidase of Human Lymphoid Cells

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SUMMARY

2′,3′-Dideoxyinosine (ddIno) is a potent and selective inhibitor of human immunodeficiency virus in human lymphoid cells and monocytes/macrophages. Earlier studies [*J. Biol. Chem.* **263**:15354 (1988)] showed that anabolism of ddIno in human lymphoid cells is mediated via an initial step of phosphorylation and subsequent amination to dideoxy-AMP via adenylosuccinate synthetase/lyase. Evidence was obtained that neither adenosine kinase nor deoxycytidine kinase is involved in the phosphorylation of this compound in human lymphoid cells. We now find that, in the presence of $MgCl_2$, KCl, and inosine-5′-monophosphate as phosphate donor, purified cytosolic 5′-nucleotidase catalyzed the phosphorylation of ddIno. Although not phosphate donors, ATP, diadenosine tetraphosphate, and glycerate-2,3-bisphosphate stimulate this phosphorylation by the nucleotidase 4–5-fold. In addition to ddIno, the antiviral nucleoside analogs 2′,3′-dideoxyguanosine and carbovir were substrates for this enzyme. The relative phosphorylation of these compounds varied with the concentration of the phosphate donor IMP. Approximate K_m values of the nucleotidase for inosine, ddIno, dideoxyguanosine, and carbovir were, respectively, 3.4, 0.5, 0.9, and 1.7 mM. Although the substrate activity of dideoxynucleosides is inefficient, it appears likely that this nucleotidase is responsible for the metabolism of these compounds to their active nucleotides, yielding antiviral activity in human lymphoid cells.

HIV has been recognized as the etiological agent of AIDS (1, 2). A number of 2′,3′-dideoxynucleosides and related compounds inhibit the infectivity and cytopathic effects of the HIV retrovirus and of these two, 3′-azido-3′-deoxythymidine and 2′,3′-dideoxycytidine, have shown anti-HIV activity in patients with AIDS (3–5). Other members of this general class are the purines ddAdo and ddIno, which are presently in Phase I clinical trials.

This laboratory has been extensively involved with studies of the metabolism of the purine dideoxynucleosides since it was shown to be effective in *in vitro* assays against HIV replication (6–8). Both ddAdo and ddIno show a high degree of selectivity against HIV in infected cells, which makes them ideal candidates for further clinical investigation (2, 5). Considerable progress has been made in elucidating the anabolism of these compounds to their putative active 5′-triphosphate species. ddAdo administered to experimental animals has a half-life on the order of a few minutes. Its principal route of metabolism appears to be deamination to its derivative ddIno (6, 8). ADA is presumably the enzyme involved in this catabolism, with a V_{max} for ddAdo very similar to that of the natural substrate adenosine (9, 10). ddIno is also a substrate of PNP, which results in the production of the inactive base hypoxanthine and a dideoxyribose phosphate sugar. However, ddIno is a poor substrate for PNP and studies show that ddAdo, although rapidly deaminated by ADA, accumulates both *in vitro* and *in vivo* as ddIno as a major product (8). This suggests that ddAdo is primarily a prodrug of ddIno and that the majority of the biological activity of ddAdo rests in the fate of its deaminated product ddIno.

Studies of the metabolism of ddIno in intact cells show that its anabolism is mediated via an initial step of phosphorylation and subsequent amination at the level of the monophosphate, via adenylosuccinate synthetase/lyase enzymes, to give ddAMP, with subsequent conversion to ddATP via cellular nucleotide kinases (6, 8). The missing link in this proposed pathway is the identity of the enzymes(s) responsible for the initial phosphorylation step. No cytosolic nucleoside kinase is known that phosphorylates inosine, guanosine, or their analogs in human cells. In contrast, it was recently found that a cytosolic 5′-nucleotidase can act as a phosphotransferase and catalyze the phosphorylation of certain inosine or guanosine

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ABBREVIATIONS: HIV, human immunodeficiency virus; ddAdo, 2′,3′-dideoxyadenosine; ddIno, 2′,3′-dideoxyinosine; carbovir, carboxylic 2′,3′-didehydro-2′,3′-dideoxyguanosine; tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide; DPG, 2,3-diphosphoglycerate; Ap4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; DTT, dithiothreitol; PNP, purine nucleoside phosphorylase; AIDS, acquired immunodeficiency syndrome; ADA, adenosine deaminase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dIno, deoxyinosine; DTT, dithiothreitol; ddGuo, 2′,3′-dideoxyguanosine; Ado, adenosine; Ino, inosine; dGuo, deoxyguanosine; dAdo, deoxyadenosine; dCyd, deoxycytidine.

analogs such as tiazofurin and the antiviral nucleoside acyclovir, albeit rather inefficiently (11, 12). Now that ddIno has been established as a major metabolite of the antiretroviral agent ddAdo and is being tested clinically in patients with AIDS, it is important to identify the biochemical pathway for its activation to the active nucleotide derivatives in target cells. This presentation characterizes the parameters affecting ddIno phosphorylation and shows that ddIno and various other dideoxynucleosides can be phosphorylated by isolated 5′-nucleotidase from human T cells.

Experimental Procedures

Materials. ddAdo and ddIno were obtained from Dr. David Johns of the Developmental Therapeutics Program, National Cancer Institute, and carbovir was provided by Dr. C. Litterst of the Development Therapeutics Branch of the National Institute of Allergy and Infectious Diseases. ddGuo was from the United States Biochemical Corp. (Cleveland, OH). Ino, dIno, dGuo, and Ap4A were from Sigma Chemical Co. (St. Louis, MO). [8-^{3}H]dAdo (20 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). [2-^{3}H]Ino (35 Ci/mmol), [8-^{3}H]dGuo (16 Ci/mmol), [8-^{3}H]carbovir (1.3 Ci/mmol), [2,8-^{3}H]ddAdo (30 Ci/mmol), and [8-^{3}H]ddGuo (3.5 Ci/mmol) were from Moravek Biochemicals (Brea, CA). [8-^{3}H]dIno and [2,8-^{3}H]ddIno were prepared by mixing 5 μCi of [8-^{3}H]dAdo or [2,8-^{3}H]ddAdo with 1.4 units of ADA (Sigma, Type X) and incubating at 37° for 10 min. [^{3}H]dIno and [^{3}H]ddIno were diluted to the appropriate concentration with 8 mM dIno or ddIno and used as is. Polyethyleneimine-cellulose thin layer chromatography plates (20 × 20 cm) with UV_{254} fluor were from Brinkmann Instruments (Westbury, NY). DTT and ATP were from Research Organics (Cleveland, OH). ACS scintillation fluid was from Amersham (Arlington Heights, IL).

Enzyme assays. Nucleoside phosphorylation by 5′-nucleotidase was done at 37° with 2.4 mM ^{3}H-labeled nucleoside (0.5 μCi) in a volume of 15 μl of buffer that contained 100 mM HEPES, pH 7.5, 50 mM $MgCl_2$, 500 mM KCl, 5 mM IMP, 3 mM DTT, 5 mM ATP, and 15 μg of Fast Flow Q/Blue Sepharose-purified nucleotidase. Phosphorylation reactions were incubated for 30 min. The assay was terminated by placing 4 μl of the reaction mixture on a PEI-cellulose thin layer chromatography plate and developing it in water until the front had migrated 10 cm. Nucleoside and nucleotide monophosphate were located by illumination of the dried plate (75°, 5 min) with UV light. Nucleotide monophosphate, as indicated by UV quenching due to IMP, remained at the origin. The monophosphate region was cut out and counted by liquid scintillation after elution in 1 ml of 0.2 M KCl and 0.1 M HCl and addition of 7 ml of ACS. Background counts and total counts per assay were determined by counting the monophosphate and nucleoside regions from a control assay without enzyme. Enzyme reaction rates were linear for the time and protein concentrations used (up to 1 mg of protein/ml for ddIno). No radioactivity was found in either the di- or triphosphate region of the thin layer chromatography plate and high pressure liquid chromatography analysis on a Partisil 10 SAX anion exchange column of the enzyme reaction product confirmed that the phosphorylation product was the monophosphate.

Purification of human lymphoblast phosphotransferase. White blood cells were obtained by leukophoresis from patients with acute lymphocytic leukemia. Contaminating red cells were removed by sedimentation in 6% hydroxyethyl starch (13). The suspension of white cells contained less than 1% red cells. Approximately 4 × 10^{11} cells were suspended in 200 ml of homogenization buffer that contained 50 mM Tris, pH 7.4, 3 mM DTT, 10% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM *O*-phenanthroline, 5 mM benzamidine, and 0.5 mg of soybean trypsin inhibitor (Sigma). Cells were homogenized with a glass Ten-Broeck homogenizer and centrifuged 15 min at 27,000 × *g* and the supernatant was centrifuged again at 105,000 × *g* for 15 min. The 105,000 × *g* supernatant was slurried with 200 ml of ion exchange resin (Fast Flow Q; Pharmacia) that was previously equilibrated with homogenization buffer. 5′-Nucleotidase was eluted from the Q-Sepharose with a 300-ml gradient of 0–0.6 M KCl in homogenization buffer, followed by isocratic elution at 0.6 M KCl after the resin was packed in a 2.6 × 30 cm column (Pharmacia XK-26). The protein was eluted at 5 ml/min and monitored at 280 nm using a Pharmacia fast protein liquid chromatography system. This procedure was adapted from the one developed by Bontemps *et al.* (13).

Phosphorylating activity was located in the column fractions using 2.4 mM Ino as the substrate. Activity corresponding to the phosphorylation of Ino was coincident with an identical assay for the hydrolysis of IMP, using [^{14}C]IMP as the substrate. 5′-Nucleotidase activity eluted at 0.6 M KCl. Fractions containing 5′-nucleotidase were pooled and slurried with 50 ml of blue Sepharose affinity gel (Reactive Blue 2 Sepharose CL-6B; Sigma) that was previously equilibrated with 50 mM Tris, pH 7.5, 3 mM DTT, 10% glycerol (Buffer A), and packed into a 2 × 15 cm column. Nonbinding proteins were eluted with Buffer A that contained 0.5 M KCl. 5′-Nucleotidase was eluted with a 2 M KCl step in Buffer A. Activity was located using the phosphorylation of Ino, as described for the Q-Sepharose elution. Fractions containing Ino-phosphorylating activity were pooled and concentrated by ultrafiltration to 5 ml, using an Amicon PM 30 membrane. The enzyme was diluted 25-fold with buffer containing 50 mM Tris, pH 7.5, 3 mM DTT, 50 mM $MgCl_2$, 500 mM KCl, and 10% glycerol and was concentrated by ultrafiltration to 2 ml. The final nucleotidase solution contained 22 mg/ml protein and was stored at −70°.

Results

On the basis of studies with mutant lymphoblast lines deficient in dCyd kinase or Ado kinase or both enzyme activities, it was shown that neither of these two enzymes is apparently required for the activation of ddAdo or ddIno to the triphosphate. As shown in Table 1, the loss of either or both Ado kinase and dCyd kinase had no detectable effect on ddATP accumulation from either nucleoside analogs.

Purification of 5′-nucleotidase from human lymphoblasts. 5′-Nucleotidase was purified from human T lymphoblasts to see whether it could catalyze the phosphorylation of ddIno. Nucleotidase and phosphotransferase activities coeluted from Q-Sepharose at 0.6 M KCl, using hydrolysis of IMP and IMP-dependent phosphorylation of Ino, respectively. This purification step removed about 75% of the protein and gave a 3-fold purification, based on Ino-phosphorylating activity (Table 2). The phosphotransferase activity was further purified by affinity chromatography on blue Sepharose, following elution with 2 M KCl. This step brought the level of protein to about 1% of the starting amount and gave an overall 23-fold purification (Table 2). The purification step provided an enzyme preparation that was free from detectable ATP-dependent nucleoside kinase activities and alkaline and acid phosphatase activity with phenylphosphate as phosphate donor at 9 and pH 5, respectively.

TABLE 1

Formation of ddATP in human CEM cells

CEM cells (5 × 10^5/ml) were incubated with 10 μM [2′,3′-^{3}H]ddAdo (2 μCi/ml). After a 6-hr incubation at 37°, cells were pelleted, briefly washed with cold saline, and extracted with 60% ice-cold methanol. Cell extracts were analyzed for radioactive nucleotides on a Partisil 10 SAX column as described (6).

Cell type	ddATP accumulation	
	ddAdo	ddIno
	pmol/10^6 cells	
CEM	1.36	1.19
CEM/dCK$^-$	1.29	1.13
CEM/AK$^-$	1.25	1.24
CEM/dCK$^-$, AK$^-$	1.31	1.22

TABLE 2

Purification of 5′-nucleotidase from human lymphoblast cells

Activities were measured with 2.4 mM Ino in the presence of 5 mM DPG and 5 mM IMP. Activity is expressed in terms of nmol of Ino phosphorylation to IMP/hr.

Step	Total protein	Total activity	Specific activity	Fold purification
	mg	*nmol/hr*	*nmol/hr/mg*	
105,000 × *g* supernatant	2,600	260,000	100	1
Fast Flow Q-Sepharose	660	174,000	290	3
Blue Sepharose	30	70,000	2,300	23

TABLE 3

Comparison of Ino phosphorylation kinetics with kinetics of dideoxynucleoside phosphorylation by 5′-nucleotidase

Assays were performed with 2 mM nucleoside, 1 mM IMP, and 5 mM ATP, using purified nucleotidase. Phosphorylation kinetics were plotted using the Enzfitter computer program. Data shown represent the best fit to the Michaelis-Menten equation. Each assay was done with 18 μg of protein and 0.2 to 2 mM nucleoside.

Substrate	K_m	V_{max}	V_{max}/K_m	V_{max}/K_m	V_{max}
	mM	*nmol/hr/mg*		*% of Ino*	*% of Ino*
Ino	3.4	49,000	14,000	100	100
ddIno	0.52	870	1,700	12	2
ddGuo	0.85	630	740	5	1.3
Carbovir	1.7	470	280	2	1

Phosphorylation of dideoxynucleosides by nucleotidase. 5′-Nucleotidase showed specificity for nucleosides containing either of the purine bases hypoxanthine and guanine but showed no phosphorylation activity for adenine-containing compounds. All three purine antiviral dideoxynucleosides, ddIno, ddGuo, and carbovir, appeared to be suitable substrates for the phosphotransferase. The efficiency of the phosphorylation of these antiviral agents was compared with that of the natural substrate Ino. As shown in Table 3, the results indicate that ddIno served as the most efficient substrate for phosphorylation in the dideoxy series. Each of the dideoxynucleosides showed a maximal velocity of phosphorylation of about 2% the rate observed with Ino.

Effect of IMP concentration on the phosphorylation rate. IMP or GMP serves as a phosphate donor with the nucleoside in the phosphotransferase reaction. Nucleoside phosphorylation kinetics were initially examined using 1 mM IMP as phosphate donor. Significant differences in the effect of IMP concentration were seen when comparing the phosphorylation of various nucleosides. The K_m values for IMP were in the range of 2 to 12 mM, depending on the nucleoside (Table 4). In general, nucleosides showing the higher rates of phosphorylation also required the highest level of IMP. This is consistent with the cyclic nature of the reaction, where more IMP is needed when the product is hydrolyzed and rephosphorylated by the same enzyme. These values were measured at a constant nucleoside concentration of 2 mM. The effect of IMP concentration from 1 to 5 mM is evident when considering the rate but a small increase in K_m for the nucleoside also occurs. The effect of IMP concentration on V_{max} for the phosphorylation of ddIno (400–2000 μM) was measured as shown in Fig. 1. The K_m values for ddIno showed a small increase from 2 to 3.3 mM, whereas the rate of phosphorylation increased 3-fold from 1 to 5 mM IMP. These data showed a K_m value of 4.4 mM for IMP when ddIno was saturating and IMP is the limiting substrate.

Activation of phosphorylation. Compounds such as ATP, Ap4A, and DPG have been identified as allosteric activators of 5′-nucleotidase activity (13–15). These compounds were also tested for activation of ddIno phosphorylation by this enzyme. ATP showed activation of ddIno phosphorylation, with a 4-fold increase achieved at 5 mM ATP. Maximal degrees of activation were about 2-fold at 1 mM IMP and 3-fold at 5 mM IMP (Fig. 2). Levels of ATP above 4 mM showed inhibition of ddIno phosphorylation that may be due to hydrolysis of ATP, during the incubation, by contaminating ATPase activity. Phosphate is a well established inhibitor of 5′-nucleotidase (16). DPG showed activation of about 2-fold from 1 to 10 mM at 5 mM IMP, without inhibition at higher concentrations (data not shown). Activation was most efficient with Ap4A. Activation of ddIno phosphorylation with Ap4A showed Michaelis-Menten saturation kinetics (Fig. 3), with K_m values of 300 μM at 5 mM IMP and 8 μM at 1 mM IMP. Ap4A gave a maximum of 3.5-fold stimulation at 600 μM and 5 mM IMP.

TABLE 4

Kinetics for IMP in the phosphotransferase reaction with nucleoside substrates

Assays were performed with 2 mM nucleoside, 5 mM DPG, and 0.5 to 5 mM IMP, using 15 μg of protein/15-μl assay, except for carbovir reactions, in which 65 μg of protein was used. Kinetic data were plotted using the Enzfitter computer program. Data represent the best fit to the Michaelis-Menten equation. Rate data (V_{max}) are presented as nmol of nucleoside phosphorylation/hr/mg at 2 mM nucleoside and saturating IMP.

Nucleoside substrate	K_m IMP	V_{max}	V_{max}/K_m	V_{max}/K_m
	mM	*nmol/hr/mg*		*% of Ino*
Ino	13	5600	430	100
dIno	16	5500	340	80
ddIno	2.1	460	220	50
dGuo	6.0	1400	230	53
ddGuo	2.1	300	140	33
Carbovir	3.9	76	20	5

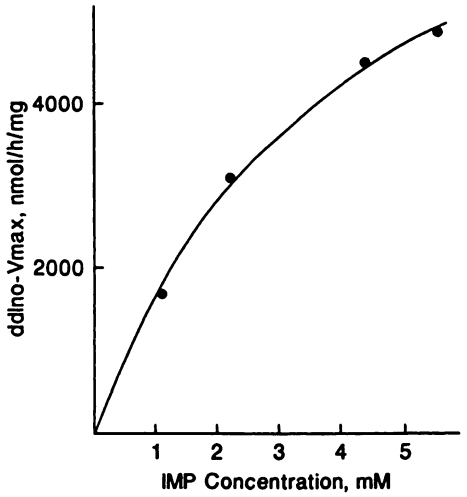

Fig. 1. Effect of IMP concentration on V_{max} for ddIno phosphorylation by 5′-nucleotidase. Assays were performed with 400–2000 μM ddIno at constant IMP concentration and 5 mM ATP at each point. Rates are expressed as V_{max} (nmol/hr/mg) for ddIno phosphorylation to IMP. Rate data were fitted to the Michaelis-Menten equation using the Enzfitter computer program. Each assay was done with 18 μg of protein. Analysis by the Enzfitter program showed a K_m for IMP of 4.4 mM and a V_{max} for ddIno phosphorylation at saturating IMP of 9000 nmol/hr/mg.

Discussion

ddAdo and the deamination product ddIno were shown by Mitsuya and Broder (2) to be equally effective in protecting

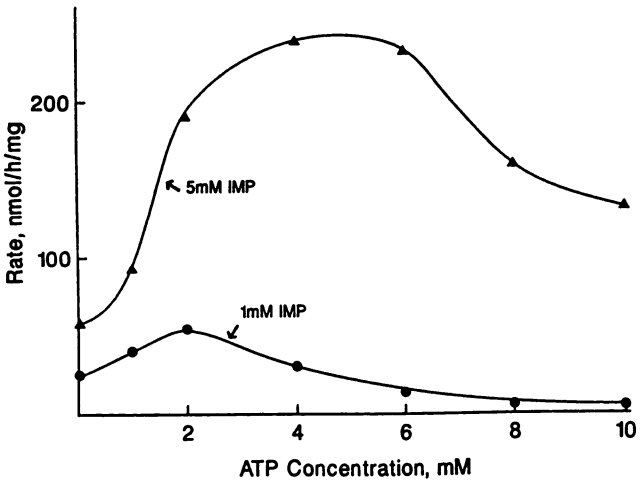

Fig. 2. Effect of ATP concentration on the rate of ddIno phosphorylation at two IMP concentrations. Assays were performed with 2.4 mM ddIno at either 1 mM IMP (●) or 5 mM IMP (▲) while the ATP concentration was varied from 0 to 10 mM. Rate data are expressed as nmol/hr/mg for each ATP concentration. Each assay contained 22 μg of protein.

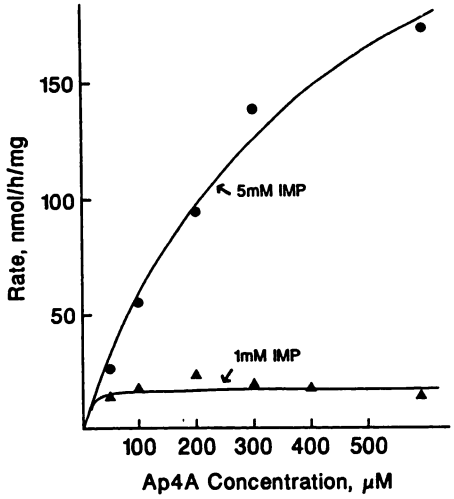

Fig. 3. Effect of Ap4A concentration on the rate of ddIno phosphorylation above the control rate (without Ap4A) at two IMP concentrations. Assays were performed with 2.4 mM ddIno at either 1 M IMP (▲) or 5 mM IMP (●) while the Ap4A concentration was varied from 0 to 600 μM. Rate data are expressed as nmol of ddIno phosphorylation/hr/mg. Data were fitted to the Michaelis-Menten equation using the Enzfitter computer program. Analysis by this method showed a K_m for Ap4A of 8 μM at 1 mM IMP and 440 μM at 5 mM IMP. Rate analysis showed activation V_{max} at saturating Ap4A of 16 nmol/hr/mg at 1 mM IMP and 300 nmol/hr/mg at 5 mM IMP. These activation rates are the rates of ddIno phosphorylation in excess of the control rate without Ap4A.

ATH8 cells from HIV cytotoxicity. Later work has shown that deamination of ddAdo is rapid in cells and the product, ddIno, accumulates as the major metabolite (5, 7). Studies presented here and elsewhere indicate that enzymes that will phosphorylate ddAdo in cell-free preparations, namely Ado kinase and dCyd kinase (Table 1), are not required for the formation of dideoxy-ATP from either ddAdo or ddIno but, rather, that an undetermined phosphorylation pathway must be involved (6, 9).

Using the enzymatic phosphorylation of Ino by the reverse reaction of 5′-nucleotidase as a probable example for ddIno activation, the phosphotransferase reaction was studied in detail. 5′-Nucleotidase has also been shown to phosphorylate the nucleoside analogs tiazofurin and acyclovir (11, 12) and a similar role for this enzyme was expected with ddIno. These results have shown that partially purified 5′-nucleotidase can phosphorylate ddIno and other antiviral drugs, ddGuo and carbovir, with apparent K_m values in the range of 0.5 to 4 mM, which is comparable to or less than that seen with tiazofurin or acyclovir. The rates of phosphorylation were 1–2% of the rate with the natural substrate Ino. These slow rates of phosphorylation are also seen for kinase activation of other antiviral dideoxynucleosides such as dideoxycytidine but may be sufficient to generate inhibitory levels of the corresponding 5′-triphosphate in cells.

The nucleotidase hydrolyzes IMP and uses IMP as a phosphate donor. The K_m value for hydrolysis is around 300 μM (13–15). Different nucleosides produce different K_m values for IMP in the phosphotransferase reaction. Nucleosides that yield monophosphates that are easily hydrolyzed by PNP seem to require more IMP and, thus, give higher K_m values. This comparison can be seen in the K_m values for IMP using Ino (a PNP-sensitive substrate) as the phosphate acceptor (12 mM), compared with ddIno (a PNP-resistant substrate) as the acceptor (2 mM). The K_m values for the nucleoside substrate, however, seem to be affected only minimally by the IMP concentration. With ddIno, the increase in K_m was from 2 to 3 mM when the IMP concentration was increased from 1 to 5 mM. The effect of IMP concentration is primarily on the velocity of the reaction, thus giving higher phosphotransferase efficiency at higher IMP concentrations.

Hydrolysis of nucleotides by cytosolic purine nucleotidase is activated by ATP, DPG, and Ap4A, all endogenous cellular metabolites (13, 14). Activation of hydrolysis results in a 12-fold increase in V_{max} with ATP and a 3-fold decrease in K_m for IMP (data not shown). Phosphotransferase activity is also activated by these cellular factors, but to a lesser extent than is hydrolysis. Phosphorylation of ddIno increases a maximum of 2-fold with ATP at 1 mM IMP. There is little change in K_m for ddIno with the addition of ATP at the maximal level of stimulation. The dinucleoside tetraphosphate Ap4A was an efficient activator, giving 3.5-fold activation of ddIno phosphorylation at 0.6 mM Ap4A and 5 mM IMP. The degree of activation also increased with the IMP level. At 1 mM IMP, 0.6 mM Ap4A activated ddIno phosphorylation 2-fold. These data are supported by studies on the activation of IMP hydrolysis by ATP and Ap4A, which suggest that the allosteric site and catalytic site for IMP interact with each other.

The combination of resistance to PNP and activation by the ubiquitous enzyme cytosolic 5′-nucleotidase provides a unique route for the utilization of therapeutic Ino analogs. ddIno shows the most efficient activation by this route among the antiviral dideoxynucleosides studied thus far. Presumably, the high therapeutic indexes shown by ddAdo and ddIno are also related to activation by nucleotidase. Further studies on the regulation of phosphotransferase activity by endogenous nucleotides could lead to the development of selective drugs for AIDS therapy that are activated following changes in cellular nucleotide pools.

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