0026-895X/88/040501-05$02.00/0

MOLECULAR PHARMACOLOGY, 34:501-505

Regulation of Adenosine Kinase by Adenosine Analogs

BERTHA B. LIN, MARY C. HURLEY, and IRVING H. FOX

Human Purine Research Center, Departments of Internal Medicine and Biological Chemistry, Clinical Research Center, The University of Michigan, Ann Arbor, Michigan, 48109

Received February 23, 1988; Accepted July 11, 1988

SUMMARY

The regulation of adenosine phosphorylation by adenosine analogs was studied using highly purified human placental adenosine kinase [ATP:adenosine 5′-phosphotransferase (EC 2.7.1.20)]. Our observations lead us to classify the analogs into three groups as follows: type I, 5′-*N*-ethylcarboxamidoadenosine and 5′-methylthioadenosine; type II, N^6-cyclohexyladenosine, N^6-L-phenylisopropyladenosine, and 2-chloroadenosine; and type III, 6-methylmercaptopurine riboside. Type I compounds are inhibitors of adenosine kinase at 0.5 μM adenosine with IC_{50} values of 25 μM for 5′-*N*-ethylcarboxamidoadenosine and 250 μM for 5′-methylthioadenosine. These compounds stimulate adenosine kinase at 5.0 μM adenosine up to a maximum of 30 to 50% above basal velocity. They are not substrates for adenosine kinase. Type II compounds are inhibitors of adenosine kinase at 0.5 μM adenosine with an IC_{50} of 220 μM for N^6-cyclohexyladenosine and 200 μM for N^6-L-phenylisopropyladenosine. These analogs also stimulate adenosine kinase at 5.0 μM adenosine. 2-Chloroadenosine, N^6-cyclohexyladenosine, and N^6-L-phenylisopropyladenosine are phosphorylated by adenosine kinase with apparent K_m values of 1, 330, and 205 μM, respectively. 6-Methylmercaptopurine riboside (type III) inhibited enzyme activity with an IC_{50} of 10 μM at 0.5 μM adenosine and 215 μM at 5 μM adenosine and is a substrate for adenosine kinase. These data are consistent with the following: (a) 2-chloroadenosine, N^6-cyclohexyladenosine, and N^6-L-phenylisopropyladenosine may not be good adenosine receptor agonists *in vivo* because they are phosphorylated into active derivatives by adenosine kinase; (b) 5′-*N*-ethylcarboxamidoadenosine and 5′-methylthioadenosine are superior candidates for adenosine receptor agonists *in vivo* because they are not phosphorylated; (c) 5′-*N*-ethylcarboxamidoadenosine, 5′-cyclohexyladenosine, N^6-L-phenylisopropyladenosine, and 2-chloroadenosine may interact with adenosine kinase at two sites on the enzyme, a catalytic site and a regulatory site; and (d) 6-methylmercaptopurine riboside may interact with the enzyme at the catalytic site only.

Adenosine has potent biological activities in mammalian cells, including inhibition of lymphoblast growth and platelet aggregation and stimulation of coronary vasodilation and hormone secretion (1). Adenosine analogs may potentially be used pharmacologically to mimic the biological activities of adenosine (1). Analogs such as N^6-cyclohexyladenosine, N^6-L-phenylisopropyladenosine, 5′-methylthioadenosine, 5′-*N*-ethylcarboxamidoadenosine, and 2-chloroadenosine (Fig. 1) have been used to study the role of adenosine receptors in mammalian cell systems. However, if these adenosine analogs are altered by phosphorylation, they may become cytotoxic. This would seriously diminish their pharmacological usefulness *in vivo*.

Other adenosine analogs have cytotoxic, anticancer, and antiviral effects. 6-Methylmercaptopurine riboside is phosphorylated into an active derivative by adenosine kinase and inhibits *de novo* purine biosynthesis (2). 5′-Methylthioadenosine is an intermediate in the polyamine metabolic pathway and is associated with cytotoxicity and inhibition of lymphoblast growth apparently without phosphorylation (Fig. 1) (3).

This work was supported by United States Public Health Service Grants 1 RO1 GM32837 and 5 MO1 RR00042 and by the University of Michigan Medical School Student Medical Summer Research Program funded through the Medical School and the National Institutes of Health Training Grants. Preliminary results are published in abstract form in *Clin. Res.* 34:1002A (1986) and *Clin. Res.* 35:820A (1987).

The objectives of this study are to determine whether adenosine analogs that are adenosine receptor ligands are substrates for adenosine kinase and to examine the regulation of adenosine kinase by adenosine analogs.

Experimental Procedures

Materials. The following reagents were obtained from Sigma Chemical Company (St. Louis, MO): Trizma base, 2-mercaptoethanol, bovine serum albumin, adenosine, 2-chloroadenosine, 5′-methylthioadenosine, N^6-L-phenylisopropyladenosine, and 6-methylmercaptopurine riboside. 5′-*N*-Ethylcarboxamidoadenosine was a gift from Dr. J. A. Bristol, Warner-Lambert Co. (Ann Arbor, MI). Sodium-ATP was purchased from Pharmacia PL Pharmaceuticals (Piscataway, NJ). N^6-Cyclohexyladenosine was obtained from Calbiochem (San Diego, CA). [8-^{3}H]Adenosine (40 Ci/mmol) was purchased from ICN Pharmaceuticals (Irvine, California), and [8-^{3}H]2-chloroadenosine (10–20 Ci/mmol) was obtained from Moravek Chemicals (Brea, CA). New England Nuclear (Boston, MA) was the source of [2,8-^{3}H]N^6-cyclohexyladenosine (13.5 Ci/mmol) and [2,8-^{3}H]5′-*N*-ethylcarboxamidoadenosine (25 Ci/mmol). [2,8-^{3}H]N^6-L-Phenylisopropyladenosine (35 Ci/mmol) and aqueous counting scintillant were purchased from Amersham (Chicago, IL). Whatman DE-81 filter paper discs were obtained through Scientific Products (McGaie Park, IL). All other chemicals were reagent grade or superior quality.

Enzyme purification. The adenosine kinase used in the study was purified from human placental cytosol by ammonium sulfate precipi-

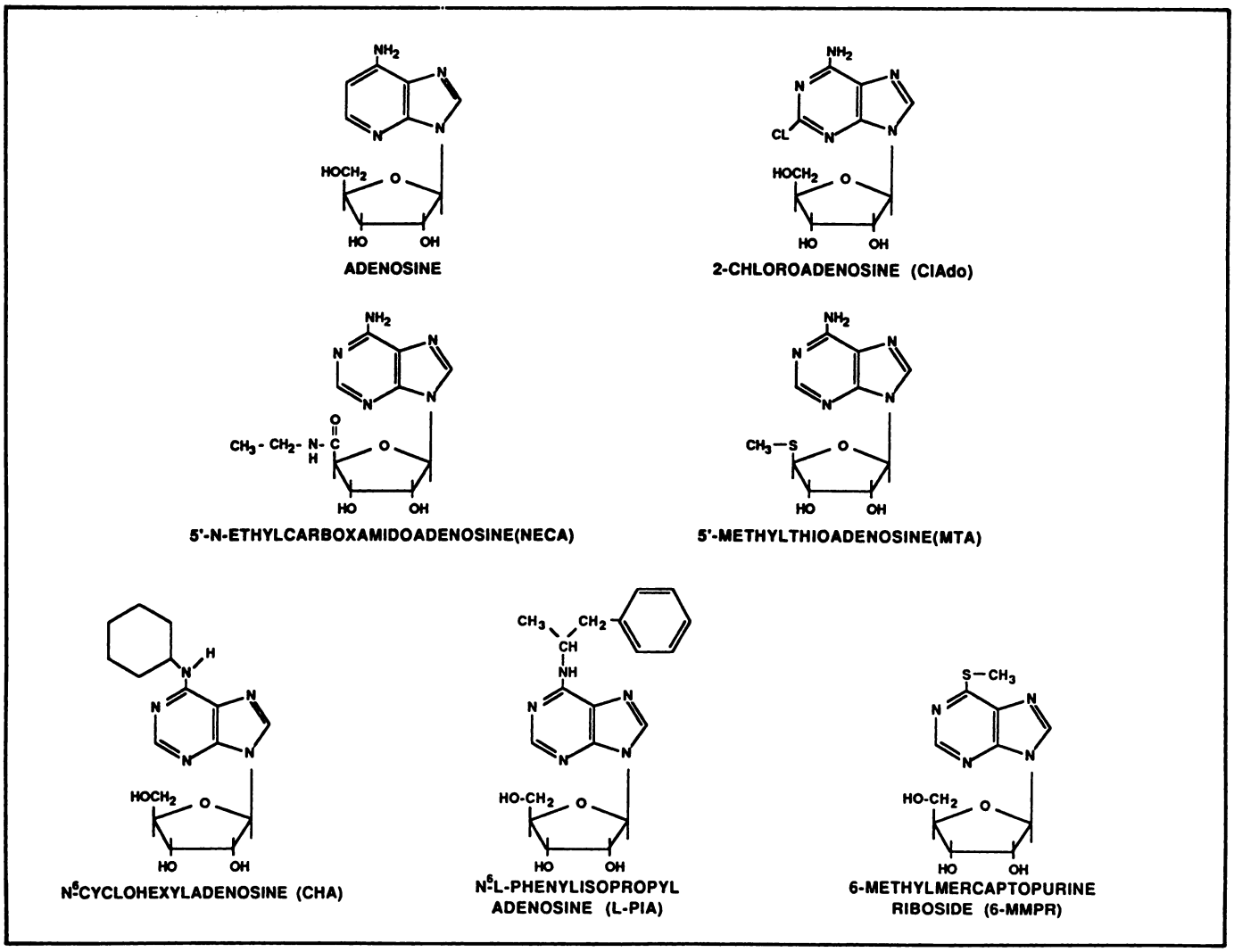

Fig. 1. Structure of adenosine and adenosine analogs. 2-Chloroadenosine (ClAdo) has a chlorine atom at the 2-position of the purine ring. 5′-*N*-Ethylcarboxamidoadenosine (*NECA*) and 5′-methylthioadenosine (*MTA*) have functional groups on the 5′-position. N^6-Cyclohexyladenosine (*CHA*), N^6-L-phenylisopropyladenosine (*L-PIA*), and 6-methylmercaptopurine riboside (*6-MMPR*) have substitutions at the 6-position of the purine ring.

tation and AMP-Sepharose 4B chromatography using a modification of the procedure by Hurley *et al.* (4). The enzyme had a specific activity of 2.15 μmol/min/mg at 10 μM adenosine and was estimated to be 1400-fold purified. This enzyme preparation is similar to our most highly purified preparation, which had a specific activity of 3.5 μmol/min/mg (5). The enzyme was kept in 50 mM Tris·HCl, pH 7.4, 50 mM 2-mercaptoethanol, and 20% glycerol and stored at −70°. Protein was measured using a modification of the assay method of Bradford (6) with bovine serum albumin as a standard.

Assay. Adenosine and adenosine analog phosphorylation was measured by determining nucleotide formation from labeled nucleosides (4, 7, 8). Adenosine phosphorylation was assayed at 37° in a 50-μl incubation medium containing 50 mM Tris·HCl, pH 7.4, 50 mM KCl, 50 mM 2-mercaptoethanol, 5% glycerol, 1 mg/ml bovine serum albumin, 1.6 mM $MgCl_2$, 1.2 mM ATP, [8-^{3}H]adenosine or [8-^{3}H]adenosine analog, and purified adenosine kinase (0.08 to 0.25 μg). Assays were incubated at 37° for 5 min and the reaction was stopped by heating for 2 min at 85°. Twenty-microliter aliquots of reaction mixture were spotted onto DE-81 filter paper discs. The discs were washed twice for 5 min each in 1 mM ammonium formate, then twice with water and once in absolute ethanol. The discs were placed in counting vials with 0.5 ml of 0.1 N HCl and 0.4 M KCl to elute the radionucleotide product and were counted in aqueous counting scintillant. The assays were linear with time and protein for the substrates and the conditions used (5, 7, 9). At 0.5 μM adenosine, a maximum of 20% of the substrate was used in the incubation media. To study the effect of adenosine analogs on adenosine phosphorylation, adenosine kinase was assayed at 0.5, 1.0, and 5.0 μM [8-^{3}H]adenosine, and adenosine analog was varied from 0 to 750 μM. Each study was performed in triplicate, and experimental values were within one standard deviation of the mean. The experiments studying the effect of adenosine analogs on adenosine phosphorylation were performed two to four times each, and representative experiments are presented in graph form.

Analysis. Double-reciprocal plots of initial velocity versus substrate concentration are linear. Kinetic data are fitted to the simple Michaelis-Menten equation (i.e., a hyperbola) by a modification of Cleland's program (10) on a Vax 11/730 minicomputer. The inhibitor constant (K_i) values are calculated from secondary plots and from the Cheng and Prusoff equation for competitive inhibitors (11, 12). For analogs that are alternate substrates for adenosine kinase, the K_i approximates a steady state constant. The use of the Cheng and Prusoff equation assumes competitive inhibition and apparent K_i values are calculated as follows:

$$K_i = \frac{IC_{50}}{1 + \frac{\text{Adenosine}}{K_m \text{ for adenosine}}}$$

The constant IC_{50} is calculated as the concentration of analog that gives 50% inhibition of adenosine phosphorylation at 0.5 μM adenosine. EC_{50} is defined as the concentration of analog that gives 50% of maximal stimulation of adenosine phosphorylation above baseline velocity achieved by the specific analog at 5 μM adenosine.

Results

Effects of adenosine on adenosine kinase activity. Adenosine kinase activity follows Michaelis-Menten kinetics at low adenosine concentrations. Initial velocity studies with variable concentrations of adenosine and ATP gave a K_m of 0.4 μM for adenosine (9). However, a decrease in adenosine kinase activity has been observed at high concentrations of adenosine (3, 9). In our studies, adenosine kinase had initial velocities of 1.83, 2.68, 3.36, 2.65, and 2.15 μmol/min/mg of enzyme at 0.1, 0.5, 1.0, 5.0, and 10.0 μM adenosine, respectively. This substrate inhibition is in agreement with earlier studies in our laboratory.

Effects of adenosine analogs on adenosine kinase activity. All adenosine analogs inhibited adenosine kinase activity at concentrations of adenosine below 1 μM, and all except 6-methylmercaptopurine riboside activated adenosine kinase

TABLE 1

Kinetic properties of adenosine analogs on adenosine kinase activity

NECA, 5′-*N*-ethylcarboxamidoadenosine; MTA, 5′-methylthioadenosine; CHA, N^6-cyclohexyladenosine; L-PIA, N^6-L-phenylisopropyladenosine; ClAdo, 2-chloroadenosine; 6-MMPR, 6-methylmercaptopurine riboside; Ado, adenosine; +, weak effect; ++, strong effect; +++, very strong effect; −, no effect; NA, not applicable; ND, not determined.

Analog	Inhibition of adenosine phosphorylation (0.5 μM Ado)	IC_{50}	K_m apparent	Stimulation of adenosine phosphorylation (5.0 μM Ado)	EC_{50}
		μM			μM
Type I					
NECA	++	25	NA	++	9
MTA	++	250	NA	++	50
Type II					
CHA	++	220	330	++	30
L-PIA	++	200	205	++	55
ClAdo	+	>750	1	+	ND
Type III					
6-MMPR	+++	10	4	−	NA

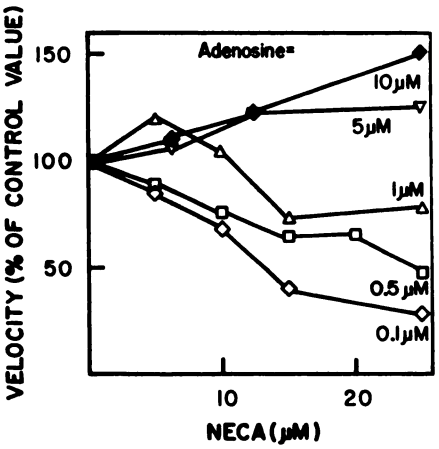

Fig. 2. Percentage of basal velocity of adenosine phosphorylation versus 5′-*N*-ethylcarboxamidoadenosine (*NECA*) concentration with 0.1 to 10 μM adenosine and 0 to 25 μM 5′-*N*-ethylcarboxamidoadenosine. Baseline initial velocities for 0.1, 0.5, 1, 5, and 10 μM adenosine are 1.83, 2.68, 3.36, 2.65, and 2.15 μmol/min/mg of enzyme, respectively.

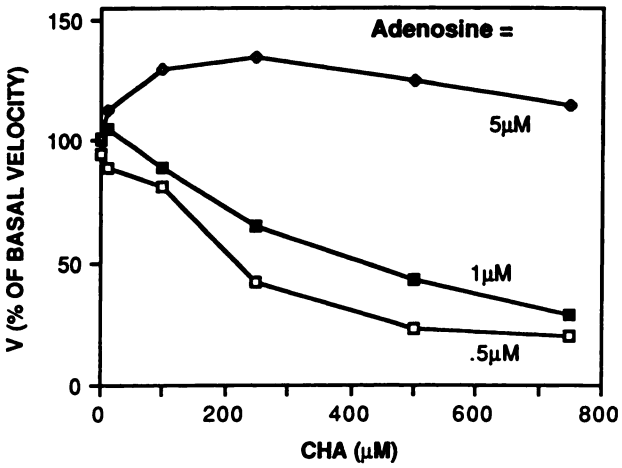

Fig. 3. Percent of basal velocity versus N^6-cyclohexyladenosine (*CHA*) concentration with 0.5 to 5 μM adenosine and 0 to 750 μM N^6-cyclohexyladenosine. Basal velocities for 0.5, 1, and 5 μM adenosine are 3.05, 3.03, and 2.22 μmol/min/mg of enzyme, respectively.

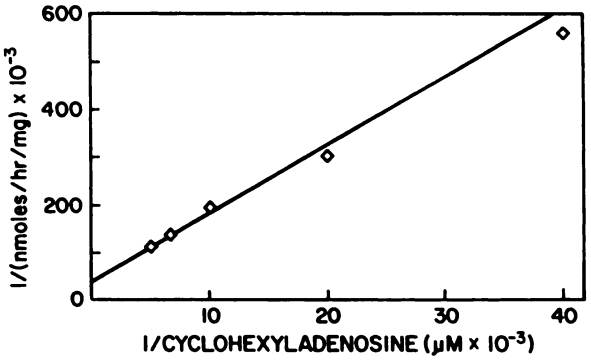

Fig. 4. Double-reciprocal plot of initial velocity study with variable N^6-cyclohexyladenosine concentrations ranging from 25 to 200 μM. The apparent K_m for N^6-cyclohexyladenosine is 330 μM.

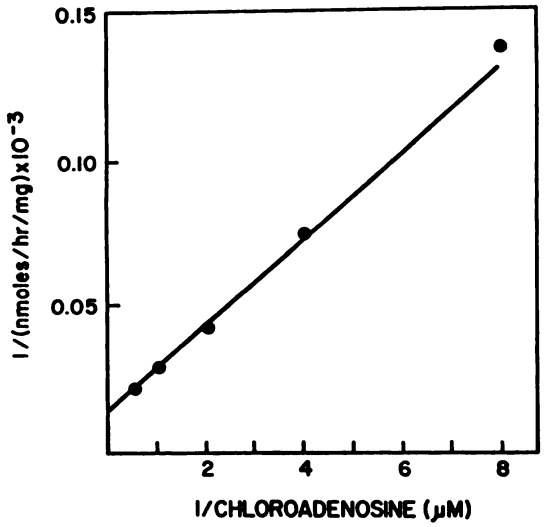

Fig. 5. Double reciprocal plot of initial velocity with variable 2-chloroadenosine concentrations from 0.125 to 2.0 μM. The apparent K_m for chloroadenosine is 1 μM.

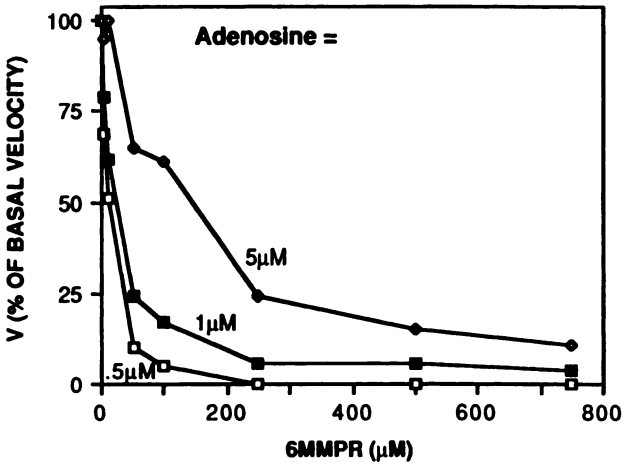

Fig. 6. Percentage of basal velocity versus 6-methylmercaptopurine riboside (*6-MMPR*) concentration with 0.5 to 5 μM adenosine and 0 to 750 μM 6-methylmercaptopurine riboside. Basal velocities at 0.5, 1, and 5 μM adenosine are 1.84, 1.80, and 1.20 μmol/min/mg of enzyme, respectively.

activity at adenosine concentrations above 1 μM. In addition, four of the analogs, N^6-cyclohexyladenosine, 2-chloroadenosine, N^6-L-phenylisopropyladenosine, and 6-methylmercaptopurine riboside, are substrates for adenosine kinase. From our observations, the adenosine analogs studied can be classified into three categories (Table 1).

Type I analogs include 5′-*N*-ethylcarboxamidoadenosine and 5′-methylthioadenosine. These analogs were found to inhibit adenosine phosphorylation at low adenosine concentrations (below 1 μM). At 0.5 μM adenosine, 5′-*N*-ethylcarboxamidoadenosine had an IC_{50} of 25 μM and an apparent K_i of 11 μM (Fig. 2). In inhibition studies with variable 5′-*N*-ethylcarboxamidoadenosine and fixed adenosine concentrations, 5′-*N*-ethylcarboxamidoadenosine was a competitive inhibitor of adenosine phosphorylation with a K_i of 7 μM. 5′-Methylthioadenosine, which is structurally similar to 5′-*N*-ethylcarboxamidoadenosine (Fig. 1), has the same effect on adenosine kinase activity, with an IC_{50} of 250 μM and an apparent K_i of 111 μM. Studies in our laboratory have previously shown that 5′-methylthioadenosine is a competitive inhibitor of adenosine phosphorylation with a K_i of 120 μM (3).

At adenosine concentrations above 1 μM, there was an increase in adenosine phosphorylation up to 50% above basal velocity, with an EC_{50} of 9 μM for 5′-*N*-ethylcarboxamidoadenosine at 5 μM adenosine. A similar effect was observed with 5′-methylthioadenosine, which had an EC_{50} of 50 μM at adenosine concentrations above 1 μM. In phosphorylation studies using radiolabeled 5′-*N*-ethylcarboxamidoadenosine or 5′-methylthioadenosine, it was found that these analogs are not substrates for adenosine kinase (3). This is due to steric blockage from the presence of functional groups at the 5′-carbon of the ribose sugar (Fig. 1).

Type II analogs are N^6-cyclohexyladenosine, N^6-L-phenylisopropyladenosine, and 2-chloroadenosine. N^6-Cyclohexyladenosine inhibited adenosine phosphorylation at 0.5 μM adenosine with an IC_{50} of 220 μM. At 5.0 μM adenosine, there was an increase in adenosine kinase activity up to 35% above basal velocity, giving an EC_{50} of 30 μM (Fig. 3). N^6-L-Phenylisopropyladenosine had much the same effect on adenosine kinase activity. At 0.5 μM adenosine, N^6-L-phenylisopropyladenosine has an IC_{50} of 200 μM and an EC_{50} of 55 μM at 5 μM adenosine. 2-Chloroadenosine exhibited similar but weaker effects on adenosine kinase, and IC_{50} and EC_{50} values could not be determined within the concentration range studied.

The phosphorylation of N^6-cyclohexyladenosine by adenosine kinase was examined using [8-^{3}H]N^6-cyclohexyladenosine. We found that N^6-cyclohexyladenosine was phosphorylated by the enzyme under conditions for adenosine phosphorylation. The double reciprocal plot of the initial velocity versus substrate concentration is a straight line and gives an apparent Michaelis-Menten constant (K_m) of 330 μM (Fig. 4). In a similar study using [8-^{3}H]N^6-L-phenylisopropyladenosine, the apparent K_m was 205 μM. [8-^{3}H]2-Chloroadenosine is also phosphorylated by adenosine kinase; the double reciprocal plot of initial velocity versus substrate concentration gives an apparent K_m of 1 μM for 2-chloroadenosine (Fig. 5).

6-Methylmercaptopurine riboside, a type III analog, inhibited the phosphorylation of 0.5 μM adenosine with an IC_{50} of 10 μM. At 1 and 5 μM adenosine, 6-methylmercaptopurine riboside was also a potent inhibitor of adenosine phosphorylation, with IC_{50} values of 23 and 215 μM, respectively (Fig. 6). In addition, 6-methylmercaptopurine riboside has been shown to be a substrate for adenosine kinase. An apparent K_m of 4 μM from secondary plots is in good agreement with the true K_m of 10 μM (2, 4).

Discussion

Adenosine kinase catalyzes the phosphorylation of adenosine to AMP and the conversion of adenosine analog substrates to their phosphorylated derivatives. Although adenosine phosphorylation follows Michaelis-Menten kinetics at low adenosine concentrations, previous studies have shown that there is a decrease in adenosine phosphorylation activity at high adenosine concentrations, a phenomenon known as substrate inhibition (9).

The mechanism for substrate inhibition of adenosine kinase has been unclear and raises the question of whether adenosine kinase has one or two adenosine binding sites. On the basis of the three types of interactions between adenosine and adenosine analogs with adenosine kinase (Table 1), we propose that adenosine kinase may have two adenosine binding sites, (a) a catalytic site with high affinity for adenosine, and (b) a regulatory site, with a lower affinity for adenosine, that inhibits the rate of adenosine phosphorylation. At low concentrations, adenosine binds only to the catalytic site and is phosphorylated to AMP, following Michaelis-Menten kinetics. At higher concentrations, adenosine binds to the regulatory site and inhibits adenosine kinase activity as the mechanism for substrate inhibition.

The properties of adenosine analog regulation of adenosine kinase may be explained by these two proposed adenosine binding sites. With the adenosine analogs studied, there is competitive inhibition of adenosine kinase activity at adenosine concentrations below 1 μM. Competitive inhibition has been established for the analogs studied by virtue of being a substrate for adenosine kinase, for which the K_i approximates a steady state constant for the analog, or by being a competitive inhibitor from initial velocity studies with variable adenosine concentrations (11). At adenosine concentrations above 1 μM, we propose that the analog competes with adenosine for binding at the adenosine regulatory site. This results in a decrease in the

amount of adenosine-mediated substrate inhibition, and the effect is seen as a net increase in adenosine phosphorylating activity. The different potencies of the analogs for inhibition of adenosine phosphorylation and stimulation of adenosine phosphorylation support the existence of a regulatory binding site different from the catalytic site. Furthermore, the fact that 6-methylmercaptopurine riboside only inhibits adenosine phosphorylation suggests that it may have major binding properties for one site only. Finally, the possibility of a second adenosine binding site on adenosine kinase, separate from the catalytic site, is strongly supported by thiol group titration and adenosine protection (13–15). The definitive establishment of a second adenosine binding site, which is associated with substrate inhibition, awaits direct structural studies.

The phosphorylation of adenosine analogs by adenosine kinase has important biological implications beyond the existence of two adenosine sites. All of the analogs studied, with the exception of 6-methylmercaptopurine riboside, are adenosine receptor agonists. 5′-Methylthioadenosine and 5′-*N*-ethylcarboxamidoadenosine are not phosphorylated and thus would not likely be incorporated into nucleic acids. However, 2-chloroadenosine, N^6-cyclohexyladenosine, and N^6-L-phenylisopropyladenosine are substrates for adenosine kinase. Evidence for 2-chloroadenosine phosphorylation in cultured cells (16) agrees with our current observations. These three analogs may theoretically become incorporated into nucleic acids, including somatic DNA. Therefore, these compounds may be hazardous to humans, a consideration that limits their pharmacological usefulness *in vivo* until such an occurrence can be evaluated. However, compounds that are phosphorylated may have potential for use as antimicrobial and antitumor agents and immunosuppressants. This is well recognized property of 6-methylmercaptopurine riboside (2) and other adenosine analogs not evaluated in the current study.

Acknowledgments

We wish to thank Nabanita Datta for assistance in determining the apparent K_m value for N^6-L-phenylisopropyladenosine, Steve Schmaltz for assistance with data analysis on the Vax 11/730 minicomputer, the nurses and patients in the Delivery Rooms of Women's Hospital, The University of Michigan, for supplying fresh placenta, and Terri Klein, Jeanne Schmaltz, and Paige Bigelow for typing the manuscript.

References

1. Fox, I. H., and W. N. Kelley. The role of adenosine and 2′-deoxyadenosine in mammalian cells. *Annu. Rev. Biochem.* **47**:655–686 (1978).
2. Henderson, J. F., A. Mikoshiba, S. Y. Chu, and I. C. Caldwell. Kinetic studies of adenosine kinase from Ehrlich ascites tumor cells. *J. Biol. Chem.* **247**:1972–1975 (1972).
3. Fox, I. H., T. D. Palella, D. Thompson, and C. Herring. Adenosine metabolism: modification by S-adenosylhomocysteine and 5′-methylthioadenosine. *Arch. Biochem. Biophys.* **215**:302–308 (1982).
4. Hurley, M. C., B. Lin, and I. H. Fox. Regulation of deoxyadenosine and nucleoside analog phosphorylation by human placental adenosine kinase. *J. Biol. Chem.* **260**:15675–15681 (1985).
5. Andres, C. M., and I. H. Fox. Purification and properties of human placental adenosine kinase. *J. Biol. Chem.* **254**:11388–11393 (1979).
6. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
7. Hurley, M. C., and I. H. Fox. Measurement of nucleoside kinase in crude tissue extracts. *Biochem. Med.* **30**:89–100 (1983).
8. Hurley, M. C., T. D. Palella, and I. H. Fox. Human placental deoxyadenosine and deoxyguanosine phosphorylating activity. *J. Biol. Chem.* **258**:15021–15027 (1983).
9. Palella, T. D., C. M. Andres, and I. H. Fox. Human placental adenosine kinase: kinetic mechanism and inhibition. *J. Biol. Chem.* **255**:5264–5269 (1980).
10. Cleland, W. W. The statistical analysis of enzyme kinetic data. *Adv. Enzymol.* **29**:1–32 (1967).
11. Dixon, M., and E. C. Webb. *Enzymes*, 3rd Ed. Academic Press, New York, 105–107, 349–353 (1979).
12. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
13. Leibach, T. K., G. I. Spiess, T. J. Neudecker, G. J. Peschke, G. Puchwein, and G. R. Hartmann. Purification and properties of adenosine kinase from dried brewer's yeast. *Hoppe-Seyler's Z. Physiol. Chem.* **352**:328–344 (1971).
14. Neudecker, T. J., and G. R. Hartmann. On the modification of adenosine kinase by thiols. *Hoppe-Seyler's Z. Physiol. Chem.* **359**:1771–1776 (1978).
15. Hawkins, C. F., and A. S. Bagnara. Adenosine kinase from human erythrocytes: kinetic studies and characterization of adenosine binding sites. *Biochemistry* **26**: 1982–1987 (1987).
16. Yamanaka, H., N. Kamatani, Y. Nishida, K. Nishioka, and K. Mikanagi. Relationship between phosphorylation and cytotoxicity of 2-chloroadenosine and 6-methylmercaptopurine riboside in human cells. *Biochem. Biophys. Acta.* **798**:291–294 (1984).

Send reprint requests to: Dr. Irving H. Fox, Clinical Research Center, A7119 University Hospital, 1500 East Medical Center Drive, Ann Arbor, Michigan, 48109-0108.