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ADENOSINE TRANSPORT BY A VARIANT OF C1300 MURINE NEUROBLASTOMA CELLS DEFICIENT IN ADENOSINE KINASE

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Summary

The uptake of adenosine by an adenosine kinase deficient variant of C1300 murine neuroblastoma cells has been studied in the absence and in the presence of erythro-9-(2-hydroxy-3-nonyl)adenine, a potent adenine deaminase inhibitor. Although 100 μM inhibitor completely blocks the metabolism of adenosine under the conditions studied, the uptake of adenosine is concentrative, i.e., the intracellular adenosine concentration exceeds the extracellular concentration. This concentrative effect decreases as the concentration of adenosine increases and is hypothesized to be due to the binding of adenosine to an intracellular component. Despite this concentrative effect, we believe that the kinetics of uptake, as determined in experiments with short (10—20 s) uptake periods, reflect the kinetics of adenosine transport by a facilitated diffusion process. This nucleoside transport system appears to be nonspecific in that the transport of adenosine is competitively antagonized by thymidine. It does not appear to be necessary to inhibit adenosine deaminase in order to study transport in these cells as the K_m for transport is not affected by the presence of erythro-9-(2-hydroxy-3-nonyl)adenine. However, erythro-9-(2-hydroxy-3-nonyl)adenine does depress the V for transport. This effect of the inhibitor is probably not due to the inhibition of adenosine deaminase as the transport of thymidine is similarly affected.

Introduction

The passage of nucleosides across cell membranes is believed to be primarily due to carrier-mediated facilitated diffusion [1]. Most studies on nucleoside

Abbreviation: EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine.

transport have measured the uptake of radiolabelled nucleoside from a bathing medium into erythrocytes or various types of cells grown in tissue culture. As nucleosides are, in general, rapidly phosphorylated once they enter cells, it has been assumed that the rate of transport rather than the rate of phosphorylation is rate limiting and that the kinetics of uptake are equal to the kinetics of transport [2]. This assumption can be circumvented by studying non-metabolizable substrates, by inhibiting the metabolism of the substrate in some way or by genetic manipulation [3]. For example, thymidine transport can be accurately studied in cells deficient in thymidine kinase or in normal cells depleted of ATP so as to prevent the phosphorylation of thymidine [4—5]. Such studies have clearly shown that earlier measurements of thymidine uptake did not in fact directly reflect thymidine transport and have brought into doubt all transport studies of metabolizable substrates.

Studies of adenosine transport are particularly prone to error due to metabolism because adenosine can be both phosphorylated by adenosine kinase and deaminated by adenosine deaminase. The present communication reports the isolation of a variant of C1300 murine neuroblastoma cells deficient in adenosine kinase and the use of this variant along with EHNA, an adenosine deaminase inhibitor, to study adenosine transport. Under the conditions studied all the adenosine uptake was accounted for by a saturable process consistent with a facilitated diffusion mechanism.

Methods

Cell line and culture conditions. Cells of the N2a clone of C1300 murine neuroblastoma were grown in plastic culture dishes in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with penicillin G (50 I.U./ml), streptomycin sulfate (10 μg/ml) and 10% γ-globulin-free serum from newborn calf. All experiments in the present communication were performed on an uncloned variant of these cells which was isolated and shown to be deficient in adenosine kinase as described in Results. These cells were generally grown in sterilized prescription bottles with approx. 150 cm^2 growing surface and were split 1 : 4 every 3 or 4 days after incubation in a 1 : 5000 solution of EDTA in isotonically buffered saline (1 : 5000 Versine; GIBCO). Cultures were fed as necessary as the media turned acid. This procedure was followed as the cells did not appear to grow well when plated at low densities.

Experimental protocol. Adenosine transport was studied using a rapid centrifugation : silicone oil technique similar to that employed by other laboratories [6—7]. Cells were removed from surface culture (EDTA in buffered saline), rinsed in serum-free medium containing 25 mM Hepes buffer, pH 7.4, and resuspended in the same medium to give a cell count of $2—5 \cdot 10^6$ cells/ml. Transport was studied at room temperature and was initiated by adding 390 μl of the cell suspension to a 12×75 mm centrifuge tube containing 10 μl [^{3}H]-adenosine. The contents of the tube were immediately transferred to a 1.5 ml microfuge tube containing 100 μl of silicone oil (8.7 ml Dow Corning 702 diffusion pump fluid + 2 ml Dow Corning 200 fluid) and transport was stopped at the appropriate time by centrifuging the sample in a microfuge (Fisher

Model 59 with the head modified to accept the 1.5 ml tubes). Duplicate samples exposed to ^3H_2O or [^{14}C]carboxylinulin were similarly processed to give measurements of the total water and extracellular water spaces of the pellets [7]. After all the samples were processed, the medium and silicone oil layers were rapidly aspirated, taking care to invert the tubes to prevent contamination of the pellets, and the tips of the tube cut off with a hot knife and allowed to fall into scintillation vials. 0.5 ml of 1 : 1 Soluene 100 (Packard Instrument Co)/isopropyl alcohol was added to each vial and the pellet dispersed with the aid of an ultrasonic probe and solubilized by a 15 min incubation at 60°C. 75 μl of glacial acetic acid followed by 6 ml scintillation mixture (ACS, Amersham Searle) were added to each vial and the radioactivity determined in a packard Liquid Scintillation Spectrometer. Samples of the appropriate supernatant media were counted under identical conditions in order to calculate the extracellular and total water spaces, the specific activity of the [^{3}H]adenosine in each particular experiment and the amount of [^{3}H]adenosine trapped in the extracellular space of the pellet. The latter value was subtraced from the total radioactivity in the pellet to give the amount of [^{3}H]adenosine transport. In 11 experiments intracellular water was calculated to be 1.29 ± 0.10 μl/10^6 cells. The extracellular (carboxylinulin) space in the same experiments was 19.2 ± 0.76% of the total water space of the pellets. Similiar values have been reported in studies on other cell types [3].

The nature of the radioactivity in the pellet was determined by centrifuging cells through 350 μl of the silicone oil layered over 100 μl of 10% trichloroacetic acid processing the samples as follows. The trichloroacetic acid-soluble material was collected dropwise after punnturing the tip of the microfuge tube with a needle. The material from 4—6 tubes was combined to form samples which were each extracted several times with water-saturated diethyl ether to extract the trichloroacetic acid and then lyophilized. Samples were redissolved in distilled water and aliquots chromatographed on Avical F containing fluorescent indicator (Analtech. Inc.) with a solvent phase of acetonitrile/*n*-butanol/0.1 M ammonium acetate/28% ammonium hydroxide (60 : 10 : 20 : 10) [8]. Carrier compounds were used to locate adenosine ($R_F \approx 0.44$) and inosine + hypoxanthine ($R_F \approx 0.20$). Nucleotides remain at the origin in this system. These three spots (adenosine, inosine + hypoxanthine and origin) were scraped into scintillation vials, the radioactivity eluted with 1 ml of 0.1 N HCl and the radioactivity determined after the addition of scintillant. A separate aliquot of each sample was counted under the same conditions in order to check that the majority of the radioactivity was recovered in the three spots.

Materials. EHNA was provided by Dr. Gertrude Elion of the Burroughs Wellcome Co. (Research Triangle, NC). [2,8,5′-^{3}H]Adenosine (60.5 Ci/mmol) was purchased from New England Nuclear. ^3H_2O and [^{14}C]carboxylinulin were from commercial sources and were gifts from Dr. Harold Feinberg of this Department. Tissue culture supplies were purchased from GIBCO (Grand Island, N.Y.). Ethylmethanesulfonate was purchased from Eastman Chemical Co. Nucleosides and related compounds were purchased from Sigma or P.L. Laboratories.

Results

Cultures of N2a cells were treated with 5 mM ethylmethanesulfonate for 2 h and 24 h later the viable cells were subcultured and exposed to 1 μM 6-methylmercaptopurine riboside. The population of cells that continued to divide was exposed to increasing concentrations of 6-methylmercaptopurine riboside until a population of cells capable of growth in 0.1 mM of this compound emerged. These cells possessed significant adenosine kinase activity and were sensitive to the cytotoxic effect of tubercidin. The selection process was therefore repeated with tubercidin and a population of cells capable of growth in 0.1 mM tubercidin was isolated. Adenosine kinase activity was not detectable in this population of cells (data not shown). All the present experiments were performed on this uncloned population of variant cells.

Table I summarizes three experiments in which the distribution of radioactivity in cells (+ trapped medium) was determined after incubation with different concentrations of adenosine for different periods of time. The control in each experiment refers to a sample of [^{3}H]adenosine which was processed with the experimental samples. Only the percentage distribution is shown, as the experiments were not quantitative and different specific activities of adenosine were used. More than half of the adenosine was metabolized in 20 s when 1 or 5 μM adenosine was studied in the absence of EHNA (Expts. 1 and 2). 10 μM EHNA completely blocked the metabolism of 5 μM adenosine during a 20 s uptake period (Expt. 2); however 3 μM EHNA failed to inhibit completely adenosine metabolism under the same conditions (data not shown). 100 μM EHNA appeared to block completely the metabolsim of adenosine at concentrations as high as 200 μM and during uptake periods as long as 240 s (Expt. 3).

TABLE I

DISTRIBUTION OF TRICHLOROACETIC ACID-SOLUBLE RADIOACTIVITY IN CELLS INCUBATED WITH [^{3}H]ADENOSINE

H + I, hypoxanthine + inosine.

	Experimental parameters			Distribution of radioactivity (% total) *		
	Adenosine (μM)	time (s)	EHNA (μM)	Origin	H + I	Adenosine
Expt. 1						
Control				1	3	93
Sample	1	20	0	37	17	32
Expt. 2						
Control				1	6	92
Sample 1	5	20	0	21	31	43
Sample 2	5	20	10	2	8	96
Sample 3	5	20	30	2	6	87
Sample 4	5	20	100	3	8	94
Expt. 3						
Control				1	4	95
Sample 1	200	20	100	2	6	92
Sample 2	200	240	100	4	5	89

* Percent of radioactivity in aliquot equal to that chromatographed.

Fig. 1 shows time courses for adenosine uptake (5 μM) in the absence and in the presence of 10 μM EHNA. The accumulation of intracellular radioactivity continued to increase rapidly throughout the time period studied when EHNA absent. 10 μM EHNA appeared to depress slightly the initial rate of uptake and to decrease markedly the subsequent rate between approx. 50 and 300 s. The secondary rate of accumulation of radioactivity in the presence of 10 μM EHNA could be due to passive diffusion or to the incomplete inhibition of adenosine metabolism by this concentration of EHNA. Even keeping this in mind, we found it surprising that the uptake appeared to be concentrative at very short time periods. We therefore performed three time-course experiments with different concentrations of adenosine in the presence of 100 μM EHNA. Under these conditions we would not expect any significant adenosine metabolism. These experiments are summarized in Fig. 2. The approximate intracellular equilibrium concentrations of adenosine were 3.4, 125 and 320 μM, respectively. Thus, although the system was concentrative, the magnitude of the concentrative effect appeared to decrease as the concentration of adenosine increased (3.4/1 = 3.4; 125/50 = 2.5; 320/200 = 1.6). A separate experiment in which 5 min uptake values for different concentrations of adenosine in the presence of 100 μM EHNA were determined showed that this was indeed the case (Fig. 3).

From the time-course experiments shown in Figs. 1 and 2 it was obvious that the uptake of adenosine was linear for only a short period of time. In the experiment summarized in Fig. 4, 20 s uptake rates of different concentrations of adenosine were determined in the presence of 100 μM EHNA. Panel A of this figure shows the hyperbolic relationship between the concentration of adenosine and the velocity of uptake; a double reciprocal plot of this data is given in panel B. The K_m and V values determined in five experiments with this

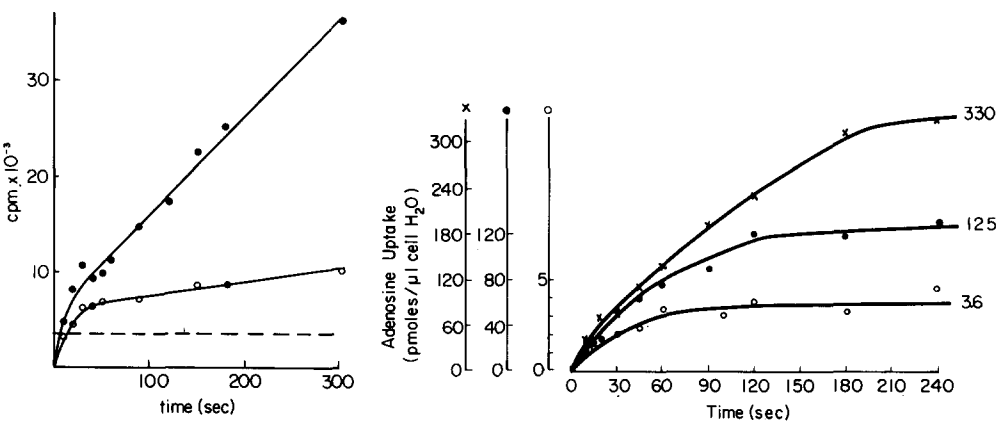

Fig. 1. Time course for the accumulation of intracellular radioactivity after incubation with 5 μM [^{3}H]-adenosine in the absence (•) or in the presence (○) of 10 μM EHNA. The dashed line indicates the amount of radioactivity at which the intracellular concentration would equal the extracellular concentration.

Fig. 2. Time course for the uptake of adenosine at 1 μM (○), 50 μM (•) and 200 μM (X); EHNA (100 μM) present. Ordinate: pmol adenosine/μl cell water. Abscissa: time.

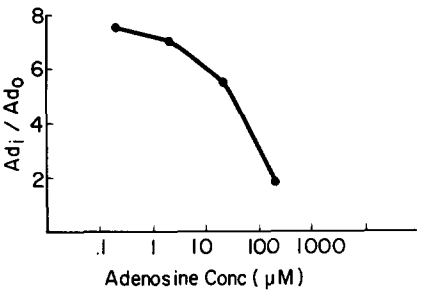

Fig. 3. Relationship between the concentrative effect of transport and the concentration of adenosine. Ordinate (Ad_i/Ad_0); intracellular [adenosine]/medium [adenosine]. Abscissa: medium [adenosine]. Incubations were for 5 min in the presence of 100 μM EHNA. Each point is the average of triplicate determinations.

experimental design were 55.7 ± 13.6 μM and 3.8 ± 0.9 pmol/μl cell water per s, respectively.

Table II summarizes two experiments in which the ability of dipyridamole and several nucleosides and related compounds to inhibit adenosine uptake was determined. In both experiments the cell suspension containing 100 μM EHNA was added to tubes containing the [^{3}H]adenosine and the test substance and the uptake terminated at 20 s. 5'-adenylylimidodiphosphate was the only compound that did not clearly inhibit uptake under the conditions studied. A separate kinetic experiment indicated that the inhibition of adenosine transport by thymidine was competitive in nature (data not shown).

We were somewhat surprised that adenine appeared to inhibit adenosine uptake, as it is generally believed that purine bases and nucleosides are transported by different systems [1]. This prompted us to be concerned that EHNA might have an effect on adenosine uptake through a mechanism other than inhibition of adenosine deaminase, i.e., that EHNA inhibits transport. A preliminary experiment with [^{3}H]thymidine showed that the simultaneous addition of 100 μM EHNA inhibited the 10 s thymidine uptake (1 μM) by approx. 50% (data not shown). Fig. 5 summarizes an experiment in which 10 s

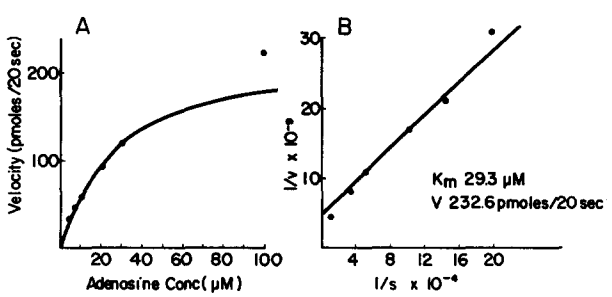

Fig. 4. Relationship between adenosine concentration and velocity of uptake. A. Plot of velocity of uptake (pmol/20 s) vs. adenosine concentration. B. Double reciprocal plot of data in part A. Each point is the average of triplicate determinations. The line in part A was drawn using the K_m (29.3 μM) and V (233 pmol/sample per 20 s) values determined from the double reciprocal plot.

TABLE II

EFFECT OF VARIOUS SUBSTANCES ON ADENOSINE UPTAKE

Cells were added to 5 μM [^{3}H]adenosine ± 100 μM of test substance (except benzylthioinosine, 30 μM); 20 s uptake

Inhibitor	cpm ± S.E. *	pmol/μl per min **	Percent inhibition
Expt. 1	6502 ± 331	17.9	—
Benzylthioinosine	2978 ± 118	6.2	65
Thymidine	4435 ± 146	11.0	39
Inosine	4039 ± 115	11.7	35
Adenine	4789 ± 41	12.0	33
Tubercidin	3732 ± 100	8.7	51
Expt. 2	3869 ± 135	9.9	—
Dipyridamole	1619 ± 50	1.4	86
γγ-Dimethylallyladenosine	1723 ± 62	1.8	82
N^6-Benzyladenosine	1485 ± 39	0.9	91
1-Methyladenosine	3134 ± 172	7.1	28
2′-*O*-Methyladenosine	2537 ± 402	4.9	51
5′-Adenylylimidodiphosphate	3605 ± 145	8.9	10
2-Chloroadenosine	1835 ± 13	2.2	78

* Absolute cpm in pellet, $n = 3$.

** Uptake corrected for trapped radioactivity and expressed as pmol/μl cell water per min.

uptakes of different concentrations of adenosine were determined in the absence of EHNA and in the presence of 10 or 100 μM EHNA. The cells were exposed to the EHNA for 5—10 min before transport was determined. It is clear from this experiment that EHNA is a noncompetitive inhibitor of adenosine transport. 10 μM EHNA did not appear to affect the 10 s transport even though we would expect it to severely, if not completely, inhibit adenosine metabolism in this experimental situation. The average K_m and V values for adenosine uptake determined in three experiments in the absence of EHNA were 24.3 ± 2.5 μM and 5.6 ± 0.4 pmol/μl cell water per s, respectively.

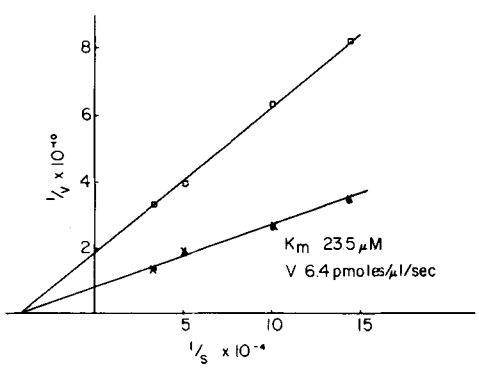

Fig. 5. Double reciprocal plot showing the effect of EHNA on adenosine transport. Ordinate $-1/v \cdot 10^{-10}$ with v = pmol adenosine transported/sample 10 s. Abscissa $-1/s \cdot 10^{-4}$ where s = molar concentration of adenosine. ●, control; X, EHNA, 10 μM; □, EHNA, 100 μM. Each point is the average of triplicate determinations.

Discussion

Most of the experiments reported in this communication were performed in the presence of 100 μM EHNA. Under this experimental condition adenosine was not metabolized to a detectable extent and the uptake of radioactivity rapidly reached a plateau. While this uptake was distinctly concentrative it was probably via a facilitated diffusion system. Concentrative uptake via facilitated diffusion in the absence of metabolism is not without precedent. Prednisone is not metabolized but is taken up into Novikoff cells to reach a concentration of approximately twice that expected for equilibrium in intracellular water [9]. It was postulated that this increased uptake was due to the lipid solibility of the prednisone and the binding of the compound to cytoplasmic receptors. The concentrative uptake of adenosine could be due to the binding of this compound to intracellular or extracellular receptors or acceptors. Extracellular binding of adenosine is unlikely to be involved as the binding of adenosine to crude neuroblastoma membranes is not antagonized by dipyridamole or thymidine and has a maximal capacity which cannot begin to account for the concentrative uptake observed (unpublished observations). Yuh and Tao [10] reported the isolation and purification of two soluble protein factors from rabbit erythrocytes which bind adenosine and cyclic AMP with a high affinity. Similar protein factors have subsequently been isolated from other tissues [11—13]. Hershfield and Kredich [14] recently reported that *S*-adenosylhomocysteine hydrolase binds adenosine and has a subunit molecular weight similar to that of the adenosine binding proteins isolated in other laboratories. They postulated that the proteins isolated in other laboratories are in fact *S*-adenosylhomocysteine hydrolase. The binding of adenosine to an intracellular protein whether it be *S*-adenosylhomocysteine hydrolase or not could explain the concentrative uptake seen in the present experiments. The finding that the concentrative effect decreases as the concentration of adenosine increases is compatible with the concentrative effect being dependent on a finite number of binding sites.

While it was generally accepted at one time that multiple specific nucleoside transport mechanisms exist, more recent experiments utilizing short uptake periods indicate that a single, rather nonspecific, transport system transports both purine and pyrimidine nucleosides [4,15—17]. It is thus not surprising that adenosine uptake was inhibited by a variety of purine nucleoside analogs as well as by thymidine and that the inhibition by thymidine appears to be competitive. The inhibition by benzylthioinosine is much greater if the cells are pre-exposed to this compound (data not shown) and thus the fairly poor inhibition in the experiment shown is, no doubt, due to the relatively slow onset of inhibition by this type of compound [18]. The surprising observation that adenine inhibits adenosine uptake led to the observation that EHNA noncompetively inhibits adenosine uptake. Thus, in retrospect, it is unfortunate that 100 μM EHNA was present in most experiments. While this concentration was necessary in some experiments in which we wished to make sure that we were completely inhibiting adenosine metabolism, it was not necessary for the study of adenosine uptake during short transport periods. The slightly lower K_m for adenosine in the series of experiments in the absence

of EHNA is probably because 10 s transport periods rather than 20 s transport periods were used in these experiments and the shorter period more accurately estimates the initial velocity of transport. The decreased velocity of uptake in the initial kinetic experiments and the analog studies in no way affects the interpretation of the experiments. While we cannot prove that the kinetic parameters which we determined were uninfluenced by the intracellular binding that we postulate to occur and account for the concentrative uptake we observe, we conclude that the adenosine kinase deficient variant of N2a cells transports adenosine by a nonspecific nucleoside transport system with a K_m for adenosine of approx. 25 μM and that the uptake kinetics accurately reflect the transport kinetics when short uptake periods are studied.

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