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Comparison of the equilibrium exchange of nucleosides and 3-*O*-methylglucose in human erythrocytes and of the effects of cytochalasin B, phloretin and dipyridamole on their transport

Peter G.W. Plagemann and Clive Woffendin

Department of Microbiology, University of Minnesota, Medical School, Minneapolis, MN 55455 (U.S.A.)

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Because of similarities in the physical and molecular properties of the nucleoside and sugar transporters of human erythrocytes and the photoaffinity labeling of the sugar transporter by 8-azidoadenosine (Jarvis et al. (1986) J. Biol. Chem. 261, 11077–11085), we have directly compared the equilibrium exchange of uridine and 3-*O*-methylglucose in these cells as measured by rapid kinetic techniques under identical experimental conditions. Both the Michaelis-Menten constant and maximum velocity were about 100-fold higher for 3-*O*-methylglucose exchange than for uridine exchange so that the first order rate constants for both transporters were about the same. When calculated on the basis of the number of nucleoside and sugar carriers per red cell estimated by equilibrium binding of nitrobenzylthioinosine and cytochalasin B, respectively, the turnover numbers for the sugar and nucleoside carriers with 3-*O*-methylglucose and uridine, respectively, as substrates were quite similar. Various sugars up to concentrations of 108 mM had no effect on the exchange of 500 μM uridine or adenosine, and uridine up to a concentration of 50 mM had no effect on the exchange of 10 mM 3-*O*-methylglucose. Adenosine, on the other hand, inhibited 3-*O*-methylglucose exchange in a concentration dependent manner, though not very effectively ($IC_{50} \approx 3$ mM). Both uridine and 3-*O*-methylglucose exchange were inhibited in a concentration dependent manner by cytochalasin B, phloretin and dipyridamole, but cytochalasin B and phloretin were 100-times more effective in inhibiting 3-*O*-methylglucose than uridine exchange, whereas the opposite was the case for the inhibition by dipyridamole.

Introduction

Mammalian cells possess a facilitated diffusion system for nucleosides with broad substrate specificity [1–3]. Two forms of the nucleoside transporter have been distinguished on the basis of their sensitivity to inhibition by nitrobenzylthioinosine [4,5]. One system is strongly inhibited by nmolar concentrations of nitrobenzylthioinosine (designated nitrobenzylthioinosine-sensitive transport), whereas the other system is only inhibited by μmolar concentrations of nitrobenzylthioinosine (designated nitrobenzylthioniosine-resistant transport). By photoaffinity labeling with ^{3}H-nitrobenzylthioinosine and reconstitution experiments, the nucleoside transporter of human erythrocytes has been identified as a 45–60 kDa, integral membrane glycoprotein [6–8]. Similar proteins have been photoaffinity-labeled with ^{3}H-

Correspondence: P.G.W. Plagemann, Department of Microbiology, 1308A Mayo Building, Box 196 UMHC, University of Minnesota Medical School, 420 Delaware Street S.E., Minneapolis, MN 55455, U.S.A.

nitrobenzylthioinosine in various other types of mammalian cells, but not in cultured cell lines that are deficient in nucleoside transport [9–11] or which express only nitrobenzylthioinosine-resistant nucleoside transport (Woffendin, C. and Plagemann, P.G.W., unpublished data). The nucleoside transporter is recovered in the same protein fraction of the human erythrocyte membrane (band 4.5) as the sugar transporter of these cells [12–14], and both therefore seem to have similar physical and molecular properties. The radiation target size of the transporters in intact red cells suggests that they may both exist as dimers [15,16] in the plasma membrane. Furthermore, both transporters are resistant to degradation by trypsin in intact red cells, but are degraded to a limited number of smaller fragments, including 20–23 kDa fragments, in unsealed ghosts, apparently due to cleavage at a sensitive cytoplasmic site(s) [17–20]. These findings and the fact that the two transporters have a similar function, namely facilitating the transfer of hydrophilic substances across the lipid bilayer, may suggest that these, and possibly other facilitated transporters of mammalian cells have similar structures and may be related at the molecular level. Indeed, it has recently been reported [21] that 8-azidoadenosine, although transported by the nucleoside transporter of human erythrocytes, efficiently photoaffinity labels the sugar transporter of these cells. Also, crossreactions between the transporters with respect to inhibition by a number of substances has been observed. For example, we found that cytochalasin B, a strong inhibitor of sugar transport [22], also inhibits the uptake of various nucleosides [23]. However, it was not unequivocally proven that the inhibition of nucleoside uptake was entirely mediated at the transport step rather than at the level of intracellular phosphorylation. Similarly, dipyridamole, a strong inhibitor of nucleoside transport [1] has been reported to affect the uptake of various sugars and of phosphate by human erythrocytes [24,25]. The amino acid sequence of the sugar transporter of human erythrocytes has recently been elucidated via sequencing of cDNAs corresponding to the band 4.5 protein [20]. The transporter is a highly hydrophobic protein, which has been postulated to traverse the lipid bilayer 12 times. Similar information is not available for the nucleoside transporter. Molecular studies of the nucleoside transporter are complicated by the fact that, based on the specific high affinity binding of ^{3}H-nitrobenzylthioinosine and [^{3}H]cytochalasin B to human erythrocytes, it amounts to only about 5% ($1.1 \cdot 10^4$ to $1.5 \cdot 10^4$ molecules/cell; Refs. 26–29) of the concentration of the sugar transporter ($2 \cdot 10^5$ to $3 \cdot 10^5$ molecules/cells; Refs. 30, 31). To assess possible relationships between the nucleoside and sugar transporters of human erythrocytes, we have directly compared the kinetics of equilibrium exchange of uridine and 3-*O*-methylglucose and the effects of various sugars, nucleosides and inhibitors of nucleoside and sugar transport on the exchange of adenosine, uridine and 3-*O*-methylglucose.

Experimental procedures

Erythrocytes from freshly drawn human blood were kindly supplied by Dr. J. Kersey (Department of Pathology, University of Minnesota) as a byproduct of lymphocyte isolation. The cells were thrice washed in cold saline containing 5 mM Tris-HCl (Tris-saline) and suspended in the same to about $6 \cdot 10^8$ to $8 \cdot 10^8$ cells/ml. The cells were enumerated with a Coulter counter.

The equilibrium exchanges of uridine, adenosine and 3-*O*-methyl-D-glucose were measured by rapid kinetic techniques as described previously [1, 32–35]. The cells were preincubated with specified concentrations of nucleosides or 3-*O*-methylglucose at 37°C for 60 min. The suspensions were cooled to 25°C and then the time-course of transmembrane equilibration of radiolabeled substrate at the same concentration as that used for preloading was measured using a dual syringe apparatus for mixing cell suspension and radiolabeled substrate solution at timed intervals (15 time points per time-course). An integrated rate equation based on the simple carrier model [1,3] was fitted to the time-courses. In experiments designed to determine the Michaelis-Menten parameters, seven substrate concentrations were used as indicated in appropriate experiments, and the parameters were extracted by least-squares regression. In other experiments, where only velocities at a single substrate concentration were of

interest, the integrated rate equation was fitted with K^{ee}, the Michaelis-Menten constant for equilibrium exchange, fixed at the value experimentally determined for the specific substrate (see Table I), and the slope at $t = 0$ was taken as the velocity of exchange (v^{ee}).

In all experiments presented, except one, suspensions of preloaded cells were mixed with the solution of radiolabeled substrate by means of the dual syringe apparatus (5 ml and 1 ml syringes) in a ratio of 7.4:1. In order to allow the assay of higher concentrations of potential inhibitors, in one experiment (see Fig. 1) the cells were preloaded with unlabeled substrate at a density of about $5 \cdot 10^9$ cells/ml and then the suspension of preloaded cells was mixed at a ratio of 1:7.4 with the solution of radiolabeled substrate containing specified concentrations of potential inhibitors. The cells were separated from the medium by centrifugation through an oil mixture and analyzed for radioactivity [32–35]. In each experiment, values of radioactivity in cell pellets were corrected for the amount of radioactivity trapped in the extracellular space, which was estimated by the use of [^{14}C]inulin [32]. Concentrations of radiolabeled intracellular substrate were expressed on the basis of intracellular water space determined by the use of ^3H_2O [32].

[5-^{3}H]Uridine and [2,8-^{3}H]adenosine were purchased from Moravek Biochemicals (Brea, CA) and 3-*O*-[^{3}H]methyl-D-glucose from ICN (Irvine, CA). Unlabeled uridine, sugars and phloretin were obtained from Sigma (St. Louis, MO) and cytochalasin B from Aldrich Chemical Co. (Milwaukee, WI). Dipyridamole (Persantin) and deoxycoformycin (Pentostatin) were gifts from Geigy Pharmaceuticals (Yonkers, NY) and Warner/Lambert (Park Davis Co., Detroit, MI), respectively.

Results and Discussion

In Table I, the kinetics parameters for uridine and 3-*O*-methylglucose exchange in human erythrocytes are compared. The values for uridine exchange are similar to those reported previously [28,33]. Those for 3-*O*-methylglucose exchange are somewhat higher than those reported for the equilibrium exchange of D-glucose in these cells (see Ref. 3). However, we measured the exchange of the non-metabolizable 3-*O*-methylglucose rather than of D-glucose and, for the first time, estimated the kinetic parameters for sugar transport in human erythrocytes by integrated rate analysis of the entire time-courses of transmembrane equilibration of the sugar. Thus, the kinetic parameters for uridine and 3-*O*-methylglucose exchange were determined in the same manner. Two striking fea-

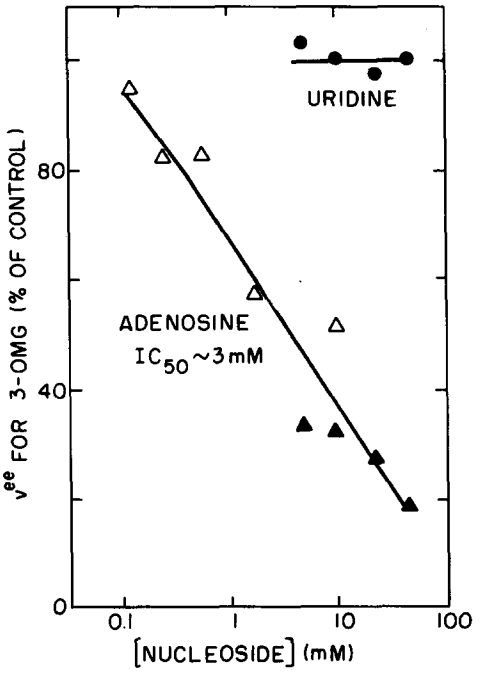

Fig. 1. Effects of uridine and adenosine on 3-*O*-methylglucose exchange in human erythrocytes. The results are from two experiments, A (empty symbols) and B (solid symbols). Suspensions of $5 \cdot 10^9$ (A) and $6 \cdot 10^8$ (B) red cells/ml of Tris-saline were equilibrated with 10 mM 3-*O*-methylglucose (3-OMG) and treated with 25 μM deoxycoformycin. Then the inward transmembrane equilibration of 10 mM 3-*O*-[^{3}H]methylglucose (10 cpm/nmol) in the presence of the indicated concentrations of uridine or adenosine was measured by rapid kinetic techniques as described under Experimental procedures. In A, the preloaded cells were mixed with the solution of radiolabeled 3-*O*-methylglucose containing uridine or adenosine, where indicated, in a ratio of 7.4:1, and in B in a ratio of 1:7.4 in order to allow the assay of higher nucleoside concentrations for inhibition. The velocities of exchange (v^{ee}) were computed by integrated rate analysis of the time-courses of radiolabel equilibration with K^{ee} fixed at 30 mM (see Table I). All values are presented as percent of the v^{ee} of the control suspensions (2.18 ± 0.29 and 2.49 ± 0.35 nmol/μl cell water per s in A and B, respectively).

TABLE I

KINETIC PARAMETERS FOR THE EQUILIBRIUM EXCHANGE OF URIDINE AND 3-*O*-METHYLGLUCOSE IN HUMAN ERYTHROCYTES AT 25 °C

The equilibrium exchanges of 50, 100, 200, 400, 800, 1600 and 3200 μM [^{3}H]uridine and of 2, 4, 8, 16, 32, 64 and 100 mM 3-*O*-[^{3}H]methylglucose in suspensions of $6 \cdot 10^8$ to $8 \cdot 10^8$ human erythrocytes/ml of Tris-saline were measured by rapid kinetic techniques and the results were analyzed by integrated rate analysis as described in Experimental procedures. K^{ee} and V^{ee} are the Michaelis-Menten constant and maximum velocity for equilibrium exchange, respectively [1,3]. The carrier turnover was calculated on the basis of $1.3 \cdot 10^4$ nucleoside carriers [26–29] and $2.5 \cdot 10^5$ sugar carriers [30,31] per red cell.

Substrate	K^{ee} (μM)	V^{ee} (μM·s^{-1})	V^{ee}/K^{ee} (s^{-1})	Carrier turnover (molecules/carrier per s)
Uridine	590 ± 60 [a]	123 ± 10	0.227 ± 0.024	410
3-*O*-Methylglucose	44100 ± 6600 [b]	9960 ± 690	0.23	1150
	30300 ± 7500 [b]	5500 ± 520	0.18	670

[a] Means ± S.E. of the mean ($n = 12$).
[b] Results from single experiment ± S.E. of the estimate.

tures were apparent from this comparison. First, both the K^{ee} and V^{ee} were about 100-times higher for 3-*O*-methylglucose exchange than for uridine exchange, so that the first order rate constants for their exchange (V^{ee}/K^{ee}) were about the same. This finding indicates that in the first order substrate concentration range for both, their rates of exchange are about the same. Whether this correlation has any physiological significance is unclear.

Second, the apparent carrier turnover was quite similar for 3-*O*-methylglucose and uridine exchange. The value for the sugar transporter was about twice that for the nucleoside transporter, but it is not clear that this difference is significant. The carrier turnover values are based on the number of molecules of nucleoside and sugar transporter per erythrocyte estimated by equilibrium binding of ^{3}H-nitrobenzylthioinosine [26–29] and of D-glucose-sensitive binding of [^{3}H]cytochalasin B [30,31], respectively, to the cells and thus any error in these estimates will affect the carrier turnover values. Furthermore, the maximum velocities for both substrates have been found to vary between populations of human red cells.

TABLE II

EFFECT OF VARIOUS SUGARS ON THE EQUILIBRIUM EXCHANGE OF URIDINE AND ADENOSINE IN HUMAN ERYTHROCYTES

The equilibrium exchanges of 500 μM [^{3}H]uridine (0.74 cpm/pmol) and of 500 μM [^{3}H]adenosine (0.2 cpm/pmol) were measured in suspensions of about $6 \cdot 10^8$ human red cells/ml by rapid kinetic techniques as described under Experimental procedures. Where indicated, sugars were added simultaneously with radiolabeled substrate. The velocities of equilibrium exchange (v^{ee}) were computed by integrated rate analysis of the time-courses of transmembrane equilibration of the radiolabeled substrate with K^{ee} fixed at 600 μM for uridine (Table I) and 100 μM for adenosine (Ref. 34, and unpublished data). Values are presented ± S.E. of the estimate. For the adenosine transport measurements, the cells were pretreated with 20 μM deoxycoformycin. No significant deamination or phosphorylation of adenosine was observed during the 60-s transport assay.

Addition		v^{ee} (pmol/μl cell water per s)	
Sugar	mM	uridine	adenosine
None		50.7 ± 4.1	47.5 ± 9.5
D-Glucose	20	51.9 ± 4.5	56.5 ± 5.1
	108	44.6 ± 4.7	46.4 ± 5.3
D-Ribose	20	57.9 ± 5.2	41.7 ± 2.2
	108	53.4 ± 5.6	42.3 ± 4.6
L-Glucose	20	48.9 ± 1.8	43.4 ± 4.7
	108	57.4 ± 4.2	37.4 ± 2.1

Next we assessed the effects of high concentrations of various sugars on uridine and adenosine exchange (Table II) and of these nucleosides on 3-*O*-methylglucose exchange (Fig. 1). The various sugars had no significant effect on uridine or adenosine exchange at least up to concentrations of 108 mM (Table II). Uridine up to a concentration of 50 mM also had no effect on 3-*O*-methylglucose exchange, but adenosine clearly inhibited

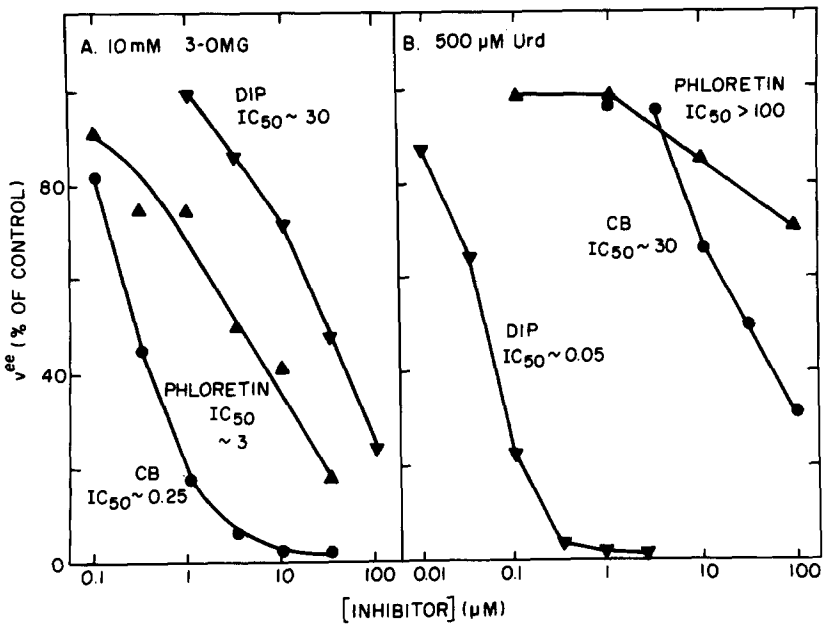

Fig. 2. Effects of cytochalasin B (CB), phloretin and dipyridamole (DIP) on the equilibrium exchange of 3-*O*-methylglucose (A) and uridine (B) in human erythrocytes. Suspensions of about $6 \cdot 10^8$ red cells/ml of Tris-saline were equilibrated with 10 mM unlabeled 3-*O*-methylglucose or 500 μM unlabeled uridine at 37 °C for about 1 h. Then the suspensions were cooled to 25 °C, supplemented with the indicated concentrations of inhibitor and the time-courses of transmembrane equilibration of 10 mM 3-*O*-[^{3}H]methylglucose and 500 μM [^{3}H]uridine were measured by rapid kinetic techniques as described under Experimental procedures. The integrated rate equation for equilibrium exchange was fitted to each time-course with K^{ee} fixed at 30 mM and 600 μM in the case of 3-*O*-methylglucose and uridine, respectively. The velocities of equilibrium exchange (v^{ee}) were calculated as the slopes of the curves at $t = 0$ and are presented as percent of the appropriate controls (1.36 ± 0.17 nmol/μl cell water per s for 10 mM 3-*O*-methylglucose and 93.9 ± 1.2 pmol/μl cell water per s for 500 μM uridine.).

3-*O*-methylglucose exchange in a concentration dependent manner, though only at relatively high concentrations (Fig. 1). The results indicate that, at best, there is only weak interaction between sugars and the nucleoside transporter and between nucleosides and the sugar transporter. This high degree of specificity of the transporters is not unexpected, even though D-ribose is a good substrate for the sugar transporter and also plays an important role in the binding of nucleosides to the nucleoside transporter. The sugar transporter is highly specific for pyranoses and pyranosides with the C1 chair form [3,36,37]; for example, it efficiently transports D-glucose and D-ribose but not their L-enantiomorphs [36]. The oxygen at carbon 1 plays an important role in the binding of sugars to the sugar transporter, but needs to be in the equatorial position (β-configuration of the pyranoses) for efficient binding. Furthermore, large substituents at carbon 1 hinder binding of the sugar to the transporter, at least in its outward configuration [37]. Nucleosides are probably poorly recognized by the sugar transporter for at least three reasons. First, the D-ribose in nucleosides is in the furanose rather than pyranose form [38]. Second, there is no oxygen linked to carbon 1, and third the substituent nucleobase at carbon 1 may sterically interfere with binding to the sugar transporter. At the molecular level, the substrate specificity of the nucleoside transporter is far less understood than that of the sugar transporter but their substrate binding sites clearly must differ greatly. That the D-ribose plays an important role in substrate recognition by the nucleoside transporter is indicated by the finding that nucleobases are generally not transported by the nucleoside transporter, with the possible exception of hypoxanthine in some cell lines [39]. The nucleobases

seem to contribute relatively little to recognition specificity of the nucleoside transporter, since it transports all natural purine and pyrimidine ribo- and deoxyribonucleosides as well as many nucleoside analogs, although not with equal efficiency [1,2,40].

It is unclear why adenosine inhibits 3-*O*-methylglucose exchange much more effectively than uridine exchange. That the inhibition of 3-*O*-methylglucose exchange by adenosine (Fig. 1) was a direct effect of adenosine and not mediated by a metabolic product of adenosine is indicated by several lines of evidence. First, adenosine phosphorylation is strongly substrate inhibited at concentrations > 1 μM (see Ref. 34). Second, adenosine deamination was blocked by the pretreatment of the cells with deoxycoformycin. Third, there was no apparent delay in the inhibition of 3-*O*-methylglucose exchange and 3-*O*-methylglucose equilibration was practically complete by 20–30 s of incubation. Fourth, the inhibition by adenosine was concentration dependent up to 50 mM, whereas all metabolic reactions involving adenosine are saturated at concentrations below 1 mM. Thus, adenosine interacts to some degree with the sugar transporter. Whether this interaction is sufficient to explain the photoaffinity-labeling of the sugar transporter by low concentrations of 8-azidoadenosine [22] is less certain, since the binding of adenosine to the sugar transporter is rather inefficient. Another possibility might be that the sugar transporter becomes photoaffinity-labeled by 8-azidoadenosine due to a close association with the nucleoside carrier as band 4.5 proteins in the red cell membrane.

Such close association of the two transporters might also explain a cross inhibition of the two transporters by various structurally unrelated substances. Fig. 2 illustrates that both uridine and 3-*O*-methylglucose exchange were inhibited by cytochalasin B, phloretin and dipyridamole, but to quite different extent. The IC_{50} values for the inhibition of uridine exchange by cytochalasin B and phloretin were about 100-times higher than for the inhibition of 3-*O*-methylglucose exchange, whereas the opposite was the case for inhibition by dipyridamole. In addition, we found that uridine exchange in two cultured cell lines (Novikoff rat hepatoma and mouse leukemia P388) was inhibited by cytochalasin B and phloretin to a similar extent as in human red cells (IC_{50} 40–100 μM) and phloretin, like dipyridamole, also inhibits phosphate flux in human erythrocytes.

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