Inhibition by nucleosides of glucose-transport activity in human erythrocytes

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The interaction of nucleosides with the glucose carrier of human erythrocytes was examined by studying the effect of nucleosides on reversible cytochalasin B-binding activity and glucose transport. Adenosine, inosine and thymidine were more potent inhibitors of cytochalasin B binding to human erythrocyte membranes than was D-glucose [IC_{50} (concentration causing 50% inhibition) values of 10, 24, 28 and 38 mM respectively]. Moreover, low concentrations of thymidine and adenosine inhibited D-glucose-sensitive cytochalasin B binding in an apparently competitive manner. Thymidine, a nucleoside not metabolized by human erythrocytes, inhibited glucose influx by intact cells with an IC_{50} value of 9 mM when preincubated with the erythrocytes. In contrast, thymidine was an order of magnitude less potent as an inhibitor of glucose influx when added simultaneously with the radioactive glucose. Consistent with this finding was the demonstration that glucose influx by inside-out vesicles prepared from human erythrocytes was more susceptible to thymidine inhibition than glucose influx by right-side-out vesicles. These data, together with previous suggestions that cytochalasin B binds to the glucose carrier at the inner face of the membrane, indicate that nucleosides are capable of inhibiting glucose-transport activity by interacting at the cytoplasmic surface of the glucose transporter. Nucleosides may also exhibit a low-affinity interaction at the extracellular face of the glucose transporter.

INTRODUCTION

Nucleosides and glucose cross the plasma membrane of human erythrocytes by functionally similar systems. Kinetic studies of nucleoside transport by human erythrocytes from fresh blood have demonstrated the presence of a kinetically symmetrical carrier with properties consistent with a simple carrier model [1,2]. In contrast, the glucose transporter of human erythrocytes is kinetically asymmetric, the extent of which decreases as the temperature increases, such that at 37 °C both K_m and V_{max} for zero-trans influx and efflux are similar [3–5]. Interestingly, the kinetic parameters obtained by using erythrocytes from outdated stored blood are significantly different from those determined with cells from fresh blood for both transport systems [1,3]. A comparison of the effects of thiol reagents and trypsin on nucleoside- and glucose-transport activity in intact erythrocytes, inside-out vesicles and disrupted membranes has provided evidence that both transporters exhibit chemical asymmetry [6–9]. Also, both transporters can be selectively inhibited by potent inhibitors that bind reversibly. Nucleoside transport is inhibited by nitrobenzylthioinosine (NBMPR) (apparent K_d 0.1–1.0 nM, 10^4 binding sites/cell), and glucose transport is blocked by cytochalasin B (apparent K_i 0.1 μ M, 2×10^5 binding sites/cell) [10–12]. At the molecular level, both transporters have been demonstrated to be ‘band-4.5’ glycoproteins (apparent M_r 65 000–45 000) that co-purify after chromatography and electrophoresis [11,13–15]. Nevertheless, the glucose and nucleoside transporters in erythrocytes are functionally distinct. For example, adult pigs possess a functional nucleoside-translocation mechanism, but are impermeable to sugars [16,17]. Inter-

estingly, plasma inosine has been demonstrated to be the major physiological energy source for adult pig erythrocytes [18,19].

A recent study [20] has demonstrated that the nucleoside 8-azidoadenosine, which is a permeant for the human erythrocyte nucleoside transporter, covalently labels the glucose transporter after exposure to u.v. light. This result, together with the previously known similarities of the two carriers, raises the possibility that nucleosides can interact with the glucose transporter. In the present study, the interaction of nucleosides with the glucose carrier was investigated by studying the effect of nucleosides on both reversible cytochalasin B-binding activity and glucose transport. It is established that nucleosides can interact with a greater affinity with the cytosolic face of the glucose carrier than with the extracellular face. A preliminary report of this study has been published [21].

MATERIALS AND METHODS

Chemicals

[G- ^{3} H]NBMPR (sp. radioactivity 35 Ci/mmol), [4(n)- ^{3} H]cytochalasin B (sp. radioactivity 17 Ci/mmol), [5,6- ^{3} H]uridine (sp. radioactivity 47.1 Ci/mmol) and D-[^{3} H]glucose (sp. radioactivity 35 Ci/mmol) were purchased from Moravek Biochemicals, Brea, CA, U.S.A., Amersham International, Amersham, Bucks., U.K., and New England Nuclear Corp., Boston, MA, U.S.A., respectively. NBMPR and NBTGR were generously given by Professor A. R. P. Paterson, Cancer Research Group (McEachern Laboratory), University of Alberta, Edmonton, Alberta, Canada. All other reagents were purchased from Sigma.

Abbreviations used: NBMPR, 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine (nitrobenzylthioinosine); NBTGR, 2-amino-6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine (nitrobenzylthioguanosine); IC_{50} , concentration causing 50% inhibition.

Cell, vesicles and membrane preparation

Fresh human blood was collected into heparin and the erythrocytes washed three times with a medium containing 140 mM-NaCl, 5 mM-KCl, 20 mM-Tris/HCl, pH 7.4 at 22 °C, 2 mM-MgCl₂ and 0.1 mM-EDTA (disodium salt). The buffy coat was discarded. Erythrocyte ghosts were prepared by osmotic lysis by the method of Dodge *et al.* [22]. Protein-depleted membranes were prepared from erythrocyte ghosts by using 0.1 mM-EDTA, pH 11.2, as described previously [13], and resuspended in 50 mM-Tris/HCl, pH 7.4.

Inside-out vesicles were prepared by the method of Steck & Kant [23] as modified by Macintyre [24]. Briefly, 4 ml of ghost membranes suspended in 5 mM-sodium phosphate, pH 8.0, at 3 mg of protein/ml, were diluted with 136 ml of prewarmed (35 °C) vesiculation medium containing 0.5 mM-sodium phosphate and 0.5 mM-EGTA, pH 8.4. This suspension was incubated for 15 min at 35 °C, and the membranes were recovered by centrifugation (30 000 *g* for 20 min). The membranes were resuspended in 4 ml of ice-cold vesiculation medium and then homogenized by using a 5 ml syringe and a no. 27-gauge needle to complete vesiculation. The vesicle suspension was diluted to 8 ml, and 2 ml aliquots were layered on to 3 ml of Dextran T-70 barrier solution [4.5% (w/v) Dextran T-70 in 0.5 mM-sodium phosphate, pH 8.0] in a 5 ml Beckman SW55 centrifuge tube and centrifuged at 100 000 *g* for 1 h at 4 °C. Sealed inside-out vesicles were recovered from the barrier interface and washed twice in ice-cold 5 mM-sodium phosphate, pH 8.0. The final vesicle pellet was resuspended to a protein concentration of 2–4 mg of protein/ml and used immediately.

Right-side-out vesicles were also prepared by the method of Steck & Kant [23]. Ghost membranes (5 ml) were diluted to 120 ml with 0.5 mM-sodium phosphate, pH 8.0, and gently stirred at 4 °C for 30 min. A portion of 200 mM-MgSO₄ was added to the suspension to give a final concentration of 0.1 mM-MgSO₄ and the membranes pelleted by centrifugation (30 000 *g* for 1 h). The membrane pellet was resuspended in 4 ml of 0.1 mM-MgSO₄/0.5 mM-sodium phosphate, pH 8.0, and homogenized as described for the inside-out vesicles. The homogenized suspension was diluted to 8 ml, and 2 ml aliquots were layered on to 3 ml of 4.5% (w/v) Dextran T-70. The sealed right-side-out vesicles were recovered after centrifugation by using the same procedure as described above, washed twice, and resuspended in sodium phosphate, pH 8.0 (2–4 mg of protein/ml).

Vesicle sidedness was established by measuring the activity of acetylcholinesterase, a marker enzyme for the extracellular membrane surface, and glyceraldehyde-3-phosphate dehydrogenase, a marker enzyme for the intracellular membrane surface, in the presence and absence of Triton X-100 [23,25]. The present vesicle preparations typically were 70–80 and 90–96% pure for inside-out and right-side-out vesicles respectively. Protein was assayed by the method of Lowry *et al.* [26], with bovine serum albumin as standard.

NBMMPR binding

[³H]NBMMPR binding to human erythrocyte protein-depleted membranes was determined by centrifugation [27]. Briefly, 50 µl of membrane (50 µg of protein) in microcentrifuge tubes were equilibrated with various

concentrations of [³H]NBMMPR (0.05–10 nM) at 22 °C for 30 min in the absence or presence of 5 µM-NBTGR as competing non-radioactive ligand (total volume 1 ml). In the case of inhibition studies, the incubation medium contained the test compound and, where necessary, D-sorbitol was added to obtain equal osmolarity conditions. After incubation the tubes were centrifuged at 12 000 *g* for 25 min. Portions of the supernatant (450 µl) were retained for radioactivity determinations, and the remaining supernatant was removed before assaying the membrane pellet for ³H radioactivity by liquid-scintillation counting. Specific NBMMPR binding is defined as the total binding minus the binding component observed in the presence of the competing ligand NBTGR.

Cytochalasin B binding

Equilibrium binding of cytochalasin B to protein-depleted erythrocyte membranes was assayed by a centrifugation method using a Beckman Airfuge [28]. Portions of membrane (0.5 ml, 0.2–0.3 mg of protein/ml) were incubated with various concentrations of [³H]-cytochalasin B (0.05–1.0 µM) in the presence of either 0.6 M-D-sorbitol or -D-glucose. When differing concentrations of sugars were used to examine their effectiveness at blocking cytochalasin B binding, the concentration of D-sorbitol was varied to maintain equal osmolarity for all conditions. After incubation for 30 min, duplicate 220 µl aliquots were removed and centrifuged at 130 000 *g* for 10 min. Portions of the supernatant (100 µl) were retained for radioactivity determinations, and the remaining supernatant was removed. The pellet was dissolved in 0.1 ml of 5% (v/v) Triton X-100 and then placed in a scintillation mini-vial. Radioactivity was determined by liquid-scintillation spectrometry, with appropriate quench correction; the individual values were within 5% of each other. High-affinity (D-glucose-sensitive) cytochalasin B binding was calculated from the difference in pellet radioactivity measured in the absence and in the presence of 0.6 M-D-glucose. Kinetic constants of D-glucose-sensitive cytochalasin B binding were determined both by non-linear least-squares fit of a single-site-saturation isotherm and by Scatchard analyses using a weighted-linear-regression computer program [37].

Nucleoside transport

The initial rates of NBMMPR-sensitive uridine uptake (0.1 mM extracellular concentration, 5 s) at 22 °C by intact erythrocytes were measured by an oil-stop procedure employing n-dibutyl phthalate [29]. The ability of sugars to cause the inward countertransport of radioactively labelled uridine was measured as previously described [30].

Glucose transport

For intact erythrocytes, initial rates of cytochalasin B-sensitive glucose uptake (5 mM extracellular concentration, 5 s incubation) were measured by a modified procedure of that used to estimate uridine-transport rates [29]. Briefly, 0.2 ml portions of cells (20% haematocrit) were rapidly mixed with an equal volume of D-[³H]-glucose at 15 °C. Incubations were terminated by removing 0.2 ml of the incubation medium and adding it to 0.75 ml of ice-cold stopping solution [1% (w/v) NaCl/1 µM-HgCl₂/1.25 mM-KI/100 µM-phloretin] [3] layered on top of 0.5 ml of ice-cold n-dibutyl phthalate in

Fig. 1. Effect of nucleosides and glucose on [^{3} H]cytochalasin B binding to protein-depleted human erythrocyte membranes

Equilibrium binding of [^{3} H]cytochalasin B (initial concn. $0.3 \mu\text{M}$) to protein-depleted human erythrocyte membranes was determined as described in the Materials and methods section. Values are expressed as percentages of control [^{3} H]cytochalasin B binding activities measured in the absence of test compound (0.97 nmol/mg of protein) and corrected for non-specific binding determined in the presence of $20 \mu\text{M}$ -cytochalasin B (0.13 nmol/mg of protein). ●, Adenosine; ○, inosine; ■, thymidine; □, uridine; ▲, D-glucose; △, L-glucose.

microcentrifuge tubes. The tubes were immediately centrifuged ($12000 g$, 15 s) and the radioactivity associated with the cell pellet determined as described previously [29]. Blank values (radioactivity that became associated with the cells during an uptake interval of zero time) were obtained by processing cell samples simultaneously exposed to radioactively labelled glucose and $10 \mu\text{M}$ -cytochalasin B at 1°C . Transport rates were calculated after subtraction of the blank (typically 20 % of the control rate).

Glucose uptake by membrane vesicles was assayed by a filtration technique. A $20 \mu\text{l}$ portion of membrane vesicles (approx. $50 \mu\text{g}$ of protein) and $20 \mu\text{l}$ of D- ^3H -glucose (10 mM , $50 \mu\text{Ci/ml}$) in 5 mM -sodium phosphate, pH 8.0, were added separately on opposite sides of a polyethylene culture tube ($16 \text{ mm} \times 100 \text{ mm}$) and preincubated at 15°C . Uptake of [^3H]glucose was initiated by continuous mixing and, after an appropriate time interval (normally 5 s), terminated by the addition of 1 ml of ice-cold stopping solution (5 mM -sodium phosphate containing $100 \mu\text{M}$ -phloretin, $1 \mu\text{M}$ - HgCl_2 , 1.25 mM -KI and $10 \mu\text{M}$ -cytochalasin B). The suspension was immediately filtered under vacuum through a $0.45 \mu\text{m}$ -pore-size nitrocellulose filter that had been prewashed with 1 ml of stopping solution. The filter was subsequently washed twice with 3 ml of ice-cold stop solution, dissolved in 4 ml of scintillation fluid and radioactivity determined. Blank values were obtained by processing samples in which ice-cold membrane vesicles pretreated with $10 \mu\text{M}$ -cytochalasin B was mixed with ice-cold glucose solution and immediately diluted with stopping solution. Vesicle glucose uptake was calculated after subtraction of these blanks. Blank values ranged from 10 to 30 % of the 5 s control transport rate.

Fig. 2. Effect of adenosine on the binding of [^{3} H]cytochalasin B to protein-depleted human erythrocyte membranes

Scatchard analysis for [^{3} H]cytochalasin B bound to D-glucose-sensitive high-affinity sites in the presence of graded concentrations of adenosine at 22°C is shown. Initial concentrations of adenosine (mM) were: ●, 0; ○, 3.3; ■, 6.7; □, 13.3.

RESULTS

Cytochalasin B binding

The effects of various concentrations of nucleosides and sugars on [^{3} H]cytochalasin B binding to alkali-stripped human erythrocyte ghosts are shown in Fig. 1. Alkali-stripped ghosts were used because these membranes retain D-glucose-sensitive cytochalasin B-binding sites and most of the high-affinity cytochalasin B-binding sites not displaceable by high concentrations of D-glucose are absent [12,31]. In agreement with previous results [32], D-glucose was shown to displace $> 90 \%$ of the saturable cytochalasin B-binding sites, with an IC_{50} value of 38 mM . L-Glucose was much less effective ($< 10 \%$ inhibition at 600 mM -L-glucose). Fig. 1 also shows that the nucleosides adenosine, inosine, thymidine and uridine effectively inhibited cytochalasin B binding, with IC_{50} values of 10, 24, 28 and $> 90 \text{ mM}$ respectively. Adenosine, inosine and thymidine were apparently more effective inhibitors of cytochalasin B binding than was D-glucose. This inhibitory effect was not due to the sugar moiety of the nucleoside, because high concentrations of D- and L-ribose (200 mM) caused less than 10 and 50 % inhibition respectively (results not shown). In other studies, cytochalasin B was shown to inhibit its own binding with an IC_{50} value of $0.8 \mu\text{M}$, whereas cytochalasin E was ineffective (no inhibition at $10 \mu\text{M}$) (results not shown).

To examine further the mechanism of adenosine inhibition of [^{3} H]cytochalasin B binding, the effect of graded concentrations of adenosine in the presence of various concentrations of [^{3} H]cytochalasin B was studied. Fig. 2 shows a Scatchard plot of D-glucose-sensitive cytochalasin B binding in the presence or absence of three concentrations of adenosine. At low concentrations of adenosine (less than 7 mM), cytochalasin B binding

Fig. 3. Inhibition of D-[^{3} H]glucose uptake in human erythrocytes by thymidine

Half the erythrocyte suspension (●) was preincubated with various concentrations of thymidine for 2 h at 22 °C, cooled to 15 °C, and then centrifuged to remove most of the extracellular medium. D-[^{3} H]Glucose (5 mM) was then added and glucose uptake determined as described in the text. The remaining erythrocytes (○) were exposed to thymidine and [^{3} H]glucose (5 mM) at 15 °C simultaneously, and the transport of [^{3} H]glucose was measured. Values are expressed as percentages of control [^{3} H]glucose influx in the absence of thymidine (470 and 453 mmol/h per litre of cells for preincubation and simultaneous conditions respectively).

Fig. 4. Time course of D-[^{3} H]glucose uptake by inside-out human erythrocyte membrane vesicles

D-[^{3} H]Glucose uptake (5 mM) by inside-out membrane vesicles was measured at 15 °C in the absence (●) or presence (○) of 10 μ M-cytochalasin B by the filtration technique described in the Materials and methods section. Values are the means for triplicate estimates.

was inhibited in an apparent competitive manner, with an inhibition constant (K_i) of 3.5 mM. Mass Law analysis by non-linear least-squares analyses of the saturable binding confirmed that adenosine concentrations of 3.3 and 6.7 mM increased the K_d with no significant effect on maximum binding. However, a higher concentration of adenosine (13.3 mM) resulted in a further increase in the K_d value, with a decrease in maximum binding, an inhibition pattern suggesting mixed inhibition. With a different batch of alkali-stripped membranes a K_i value of 2.5 mM for adenosine inhibition was observed. Similar experiments also demonstrated that thymidine (concentration range 0–20 mM) competitively inhibited D-glucose-sensitive cytochalasin B binding (K_i value of 5.0 mM; mean result for two experiments).

D-Glucose transport activity

In a complementary approach, the effect of thymidine, a non-metabolized nucleoside in human erythrocytes, on D-glucose transport (extracellular concentration 5 mM) was investigated. When thymidine and [^{3} H]glucose were added simultaneously, thymidine inhibited glucose influx when present at high concentrations (approx. 50% inhibition at 90 mM-thymidine; see Fig. 3). In contrast, preincubation of cells with thymidine for 2 h at 37 °C to allow equilibration, followed by rapid removal of the extracellular thymidine so that most of the thymidine was only present inside the erythrocyte, resulted in marked inhibition of glucose influx (IC_{50} value of 9 mM; Fig. 3). These results suggest that thymidine is interacting at both the extracellular and the cytoplasmic surface of the glucose carrier, with a relatively greater affinity of interaction with the cytoplasmic face than with the extracellular face. An alternative explanation, namely that the extracellular thymidine is inhibiting glucose transport because of intracellular accumulation of thymidine during the 5 s incubation period, is not valid. The maximum intracellular thymidine concentrations achievable after 5 s at extracellular thymidine concentrations sufficient to saturate the nucleoside carrier are at the most 0.2 mM [1,2], a concentration that produces no significant inhibition of glucose transport. Moreover, the addition of 10 μ M-NBMPR to inhibit thymidine influx had no effect on the ability of extracellular thymidine to compete with glucose for entry (results not shown). Additional experiments demonstrated that NBMPR had no effect on [^{3} H]glucose influx by erythrocytes, even at high concentrations such as 10 μ M, a concentration 10^4 -fold greater than the K_i value for inhibition of nucleoside transport [11]. Cytochalasin E (10 μ M) was also ineffective (< 5% inhibition). In contrast, cytochalasin B, as expected, inhibited the zero-trans influx of 5 mM-glucose with an IC_{50} value of 0.6 μ M.

As a further test of the greater affinity of nucleosides with the cytosolic face of the glucose carrier, the ability of thymidine to inhibit glucose transport in membrane vesicles prepared from human erythrocyte ghosts was investigated. Fig. 4 shows the time course of glucose uptake (extravesicular concentration 5 mM) by inside-out vesicles at 15 °C in the presence and absence of cytochalasin B (10 μ M). Inside-out vesicles are capable of a rapid glucose uptake that was sensitive to inhibition by cytochalasin B; the residual glucose uptake in the presence of cytochalasin B was 7% of the control value at 5 s. Similar time courses for glucose uptake by right-side-out vesicle preparations were obtained and, in

Fig. 5. Comparison of the effect of thymidine on D-[^{3} H]glucose uptake by inside-out and right-side-out membrane vesicles

D-[^{3} H]Glucose uptake (5 mM) by membrane vesicles was measured at 15 °C (5 s incubation) in the presence of various concentrations of thymidine. Values are means for four separate experiments and are expressed as percentages of glucose uptake measured in the absence of thymidine [10.4 ± 3.1 and 25.1 ± 7.5 μ mol/h per mg of protein for right-side-out (●) and inside-out (○) membrane vesicles respectively].

subsequent studies, estimates of initial glucose-transport rates by both vesicle preparations were determined with a 5 s incubation period.

Fig. 5 compares the effect of thymidine on glucose uptake by inside-out and right-side-out vesicles. Glucose uptake by inside-out vesicles compared with right-side-out vesicles was 2-fold more susceptible to thymidine inhibition under identical experimental conditions, supporting the suggestion that thymidine has a greater affinity for the glucose transporter at the cytosolic face. The difference between thymidine inhibition of glucose transport in inside-out versus right-side-out vesicles is much smaller than that apparent for intracellular versus extracellular nucleoside in the experiment of Fig. 3. However, it should be noted that the inside-out vesicles are less pure (70–80 %) than the right-side-out vesicles (90–96 %), and thus the true inhibition curve in Fig. 5 for a 100 % preparation of inside-out vesicles is likely to be shifted to the left, thereby increasing the difference in transport inhibition between inside-out and right-side-out vesicles.

To investigate further the mechanism of inhibition of glucose transport by thymidine and the possibility that thymidine may be a poor permeant for the glucose transporter, the countertransport of [^{3} H]glucose after preloading human erythrocytes with thymidine and D-galactose was compared (Fig. 6). Preincubation of human erythrocytes with 50 mM-D-galactose resulted in a transient accumulation of [^{3} H]glucose. In contrast, the time course for [^{3} H]glucose uptake by cells incubated previously with thymidine (50 mM) showed inhibition of the initial rate of uptake, but the final equilibrium value was unaffected. Similar studies using [^{3} H]uridine showed that preincubation of human erythrocytes with 5 mM-thymidine resulted in a 3-fold transient accumulation of

Fig. 6. Countertransport of D-glucose and uridine after preincubation of human erythrocytes with sugars and nucleosides

(a) Human erythrocytes were 'preloaded' with 50 mM-D-galactose (■), 50 mM-thymidine (○) and incubation medium (●), washed free of the extracellular test compound, and then resuspended in D-[^{3} H]glucose (4 mM). The appearance of cell-associated ^{3} H at 15 °C was measured as a function of time. (b) Human erythrocytes were 'preloaded' with 5 mM-thymidine (○), 25 mM-D-glucose (□), incubation medium (●) and 5 μ M-NBTGR (▲), washed free of extracellular test compound, and then resuspended in [^{3} H]uridine (0.2 mM). The appearance of cell-associated [^{3} H]uridine at 22 °C was measured as a function of time. Cells preincubated with 50 mM-D-galactose gave values identical with those obtained with glucose (results not shown).

[^{3} H]uridine compared with the control (Fig. 6). In contrast, preincubation of cells with 50 mM-D-galactose or 25 mM-D-glucose exhibited time courses for [^{3} H]uridine uptake indistinguishable from those with cells not exposed to sugars (Fig. 6).

Table 1. Comparison of the effect of cytochalasins on uridine and glucose influx

Human erythrocytes were preincubated with various concentrations of cytochalasins for 15 min at 22 °C. Initial rates of uridine influx (0.1 mM, 22 °C) and D-glucose influx (5 mM, 15 °C) were determined as described in the Materials and methods section. Mean control values for uridine and glucose influx were 34 and 389 mmol/h per litre of cells respectively. Values represent means for triplicate estimates from two different donors with an S.D. of less than 5% for the triplicate estimates. Abbreviation: N.D., not determined.

Inhibitor	Concn. (μ M)	Transport activity (% of control)	
		Uridine	Glucose
Cytochalasin B	1	95	26
	5	82	N.D.
	10	70	6
Dihydrocytochalasin B	1	70	92
	5	36	N.D.
	10	16	75
Cytochalasin E	1	99	106
	5	98	101
	10	42	100

NBMPR binding and uridine transport

The finding that nucleosides can effect the transport of glucose raises the possibility that sugars can interact with the NBMPR-sensitive nucleoside carrier. Therefore the effect of D-glucose, L-glucose, cytochalasin B and cytochalasin E on 0.1 nM-[³H]NBMPR binding to protein-depleted human erythrocyte membranes was investigated. No effect was observed with these compounds, even at high concentrations (500 mM for D- and L-glucose, and 25 μ M for the cytochalasins). In another study, the NBMPR-binding parameters of human erythrocyte ghosts in the absence and presence of 680 mM-D-glucose were shown to be similar (apparent K_d 0.76 $\pm$ 0.15 and 0.71 $\pm$ 0.18 nM; B_{max} values 25.9 $\pm$ 1.8 and 27.4 $\pm$ 2.6 pmol of NBMPR bound/mg of protein respectively). Similarly D- and L-glucose had no effect on 0.1 mM-uridine influx (less than 10% inhibition at 100 mM). In contrast, certain cytochalasins were able to inhibit uridine influx. Table 1 demonstrates that dihydrocytochalasin B, a poor inhibitor of glucose transport [32–34], was the most potent inhibitor of uridine influx of the cytochalasins tested (80% inhibition at 10 μ M). Cytochalasin B also inhibited uridine influx, although with a diminished sensitivity compared with inhibition of glucose transport (30% inhibition at 10 μ M). In contrast, cytochalasin E was without effect.

DISCUSSION

This study demonstrated that nucleosides inhibited D-glucose-sensitive cytochalasin B binding and D-glucose transport in human erythrocytes. Uptake of glucose by human erythrocytes was approx. 10-fold more sensitive to inhibition by thymidine when the nucleoside was mainly present at the cytoplasmic, as opposed to the

extracellular, surface. Consistent with this finding was the demonstration that glucose uptake by inside-out membrane vesicles was also more susceptible to inhibition by thymidine than when a vesicle preparation consisting of right-side-out membrane vesicles was used. In addition adenosine, inosine and thymidine and, to a lesser extent, uridine, were shown to inhibit D-glucose-sensitive cytochalasin B binding. However, the mode of inhibition of cytochalasin B binding by nucleosides is complex. At low concentrations (less than 7 mM and 25 mM for adenosine and thymidine respectively), adenosine and thymidine acted as apparent competitive inhibitors of D-glucose-sensitive cytochalasin B binding, with K_i values of 3.0 and 5.0 mM respectively. In comparison, the K_i value for D-glucose inhibition of cytochalasin B binding to human erythrocyte membranes has been shown previously to range from 30 to 60 mM [12,32,34]. Higher concentrations of adenosine resulted in a mixed type of inhibition, suggesting that adenosine is also interacting with the erythrocyte membrane at a site distinct from the high-affinity cytochalasin B-binding site. Cytochalasin B has been postulated to bind with high affinity (apparent K_d 0.1 μ M) to the glucose carrier at the inner face of the membrane [35,36]. Thus the findings that (i) nucleosides are more potent inhibitors of glucose transport when present at the cytoplasmic surface as opposed to the extracellular surface and (ii) low concentrations of adenosine and thymidine inhibit D-glucose-sensitive cytochalasin B binding in a competitive manner suggest that the nucleosides are acting with greater affinity at the inner surface of the membrane than at the extracellular face. Cytochalasin B and nucleosides might bind to the same specific site, or alternatively to different sites, causing conformational changes that prevent one another from binding. The present data do not exclude the possibility that nucleosides also interact with low affinity at the extracellular face of the glucose transporter. Extracellular nucleosides are capable of inhibiting glucose transport (Figs. 3 and 5), and the finding that high concentrations of adenosine result in mixed inhibition of cytochalasin B binding may be due to adenosine binding to the extracellular surface with low affinity. The failure of thymidine to cause the countertransport of D-glucose suggests that thymidine is not a permeant for the glucose carrier.

The specific inhibitor of nucleoside transport, NBMPR, had no effect on either glucose uptake or high-affinity cytochalasin B binding. Similarly, high concentrations of D- and L-glucose (500 mM) and cytochalasin B and E (25 μ M) also failed to inhibit NBMPR binding by human erythrocyte membranes. Glucose and galactose were also without effect on the influx of uridine by human erythrocytes when present at either the inner or outer membrane surface. In contrast, various cytochalasin derivatives were able to inhibit uridine influx in an apparently selective manner. Cytochalasin E was without effect, but dihydrocytochalasin B caused 50% inhibition of uridine influx at concentrations less than 5 μ M, a concentration that had little effect on glucose influx. Cytochalasin B also inhibited uridine influx, but with a sensitivity of about 100-fold less than that observed for inhibition of D-glucose uptake. These results further support the use of NBMPR and cytochalasin B as specific probes of the NBMPR-sensitive facilitated-diffusion nucleoside transporter and the facilitated-diffusion glucose carrier respectively. However, high

concentrations of cytochalasin B ($> 5 \mu\text{M}$) should be avoided, because at these concentrations inhibitory effects of cytochalasin B on the nucleoside carrier are apparent.

In conclusion, the present results demonstrate that nucleosides can interact with the glucose transporter of human erythrocytes at the inner face of the membrane. This interaction is unlikely to have any physiological significance, because the plasma concentration of nucleosides, and hence the maximum intracellular concentration in the erythrocyte, do not exceed $10 \mu\text{M}$ [19,38], a value that is 300–2800-fold less than the IC_{50} values for nucleoside inhibition of glucose transport. The present results further indicate the structural similarities between the glucose and nucleoside transporters in human erythrocytes.

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REFERENCES

- Jarvis, S. M., Hammond, J. R., Paterson, A. R. P. & Clanachan, A. S. (1983) *Biochem. J.* **210**, 457–461
- Plagemann, P. G. W., Wohlhueter, R. M. & Erbe, J. (1982) *J. Biol. Chem.* **257**, 12069–12074
- Weiser, M. B., Razin, M. & Stein, W. D. (1983) *Biochim. Biophys. Acta* **727**, 379–388
- Brahm, J. (1983) *J. Physiol. (London)* **339**, 339–354
- Lowe, A. G. & Walmsley, A. R. (1986) *Biochim. Biophys. Acta* **857**, 146–154
- Jarvis, S. M. & Young, J. D. (1982) *J. Physiol. (London)* **324**, 47–66
- Tse, C.-M., Wu, J.-S. R. & Young, J. D. (1985) *Biochim. Biophys. Acta* **818**, 316–324
- Masiak, S. J. & LeFevre, P. G. (1977) *Biochim. Biophys. Acta* **465**, 371–377
- Van Steveninck, J., Weed, R. I. & Rothstein, A. (1965) *J. Gen. Physiol.* **48**, 617–632
- Cass, C. E., Gaudette, L. A. & Paterson, A. R. P. (1974) *Biochim. Biophys. Acta* **345**, 1–10
- Jarvis, S. M. & Young, J. D. (1987) *Pharmacol. Ther.* **32**, 339–359
- Wheeler, T. J. & Hinkle, P. C. (1985) *Annu. Rev. Physiol.* **47**, 503–517
- Jarvis, S. M. & Young, J. D. (1981) *Biochem. J.* **194**, 331–339
- Baldwin, S. A., Baldwin, J. M. & Lienhard, G. E. (1982) *Biochemistry* **21**, 3836–3841
- Wu, J. S., Jarvis, S. M. & Young, J. D. (1983) *Biochem. J.* **214**, 995–997
- Kim, H. D. & McManus, T. J. (1971) *Biochim. Biophys. Acta* **230**, 1–11
- Young, J. D., Paterson, A. R. P. & Henderson, J. F. (1985) *Biochim. Biophys. Acta* **842**, 214–224
- Zeidler, R. B., Metzler, M. H., Moran, J. B. & Kim, H. D. (1985) *Biochim. Biophys. Acta* **838**, 321–328
- Young, J. D., Jarvis, S. M., Clanachan, A. S., Henderson, J. F. & Paterson, A. R. P. (1986) *Am. J. Physiol.* **251**, C90–C94
- Jarvis, S. M., Young, J. D., Wu, J.-S. R., Belt, J. A. & Paterson, A. R. P. (1986) *J. Biol. Chem.* **261**, 11077–11085
- Jarvis, S. M. (1986) *Proc. Int. Union Physiol. Sci.* **16**, 543
- Dodge, J. T., Mitchell, C. & Hanahan, D. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- Steck, T. L. & Kant, J. A. (1974) *Method Enzymol.* **31**, 172–179
- Macintyre, J. D. (1982) in *Red Cell Membranes – A Methodological Approach* (Ellory, J. C. & Young, J. D., eds.), pp. 199–217, Academic Press, London
- Mawby, W. J. & Findlay, J. B. (1978) *Biochem. J.* **172**, 605–611
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Jarvis, S. M. & Young, J. D. (1980) *Biochem. J.* **190**, 373–376
- Jarvis, S. M., Ellory, J. C. & Young, J. D. (1986) *Biochim. Biophys. Acta* **855**, 312–315
- Jarvis, S. M., Martin, B. W. & Ng, A. S. (1985) *Biochem. Pharmacol.* **34**, 3237–3241
- Jarvis, S. M., Chapman, J. D., Ngan-Lee, J., Rutledge, K. A., Barr, P. J. & Paterson, A. R. P. (1982) *Cancer Res.* **42**, 4358–4363
- Widdas, W. F. (1980) *Curr. Top. Membr. Transp.* **14**, 165–223
- Jung, C. Y. & Rampal, A. L. (1977) *J. Biol. Chem.* **252**, 5456–5463
- Rampal, A. L., Pinkofsky, H. B. & Jung, C. Y. (1980) *Biochemistry* **19**, 679–683
- Lin, S. & Spundich, J. A. (1974) *J. Biol. Chem.* **249**, 5779–5783
- Basketter, D. A. & Widdas, W. F. (1978) *J. Physiol. (London)* **278**, 389–401
- Deves, R. & Krupka, R. M. (1978) *Biochim. Biophys. Acta* **510**, 339–348
- Barlow, R. B. (1983) *Biodata Handling with Microcomputers*, Elsevier Biosoft, Cambridge
- Gerlach, E. & Becker, B. F. (1987) *Topics and Perspective in Adenosine Research*, Springer-Verlag, Berlin

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