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Adenosine Transport and Nitrobenzylthioinosine Binding in Human Placental Membrane Vesicles from Brush-Border and Basal Sides of the Trophoblast

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Summary. The nucleoside transport activity of human placental syncytiotrophoblast brush-border and basal membrane vesicles was compared. Adenosine and uridine were taken up into an osmotically active space. Adenosine was rapidly metabolized to inosine, metabolism was blocked by preincubating vesicles with 2′-deoxycoformycin, and subsequent adenosine uptake studies were performed in the presence of 2′-deoxycoformycin. Adenosine influx by brush-border membrane vesicles was fitted to a two-component system consisting of a saturable system with apparent Michaelis-Menten kinetics (apparent K_m approx. 150 μM) and a linear component. Adenosine uptake by the saturable system was blocked by nitrobenzylthioinosine (NBMPR), dilazep, dipyridamole and other nucleosides. Inhibition by NBMPR was associated with high-affinity binding of NBMPR to the brush-border membrane vesicles (apparent K_d 0.98 ± 0.21 nM). Binding of NBMPR to these sites was blocked by adenosine, inosine, uridine, thymidine, dilazep and dipyridamole, and the respective apparent K_i values were 0.23 ± 0.012, 0.36 ± 0.035, 0.78 ± 0.1, 0.70 ± 0.12 (mM), and 0.12 and 4.2 ± 1.4 (nM). In contrast, adenosine influx by basal membrane vesicles was low (less than 10% of the rate observed with brush-border membrane vesicles under similar conditions), and hence no quantitative studies of adenosine uptake could be performed with these vesicles. Nevertheless, high-affinity NBMPR binding sites were demonstrated in basal membrane vesicles with similar properties to those in brush-border membrane vesicles (apparent K_d 1.05 ± 0.13 nM and apparent K_i values for adenosine, inosine, uridine, thymidine, dilazep and dipyridamole of 0.14 ± 0.045, 0.54 ± 0.046, 1.26 ± 0.20, 1.09 ± 0.18 mM and 0.14 and 3.7 ± 0.5 nM, respectively). Exposure of both membrane vesicles to UV light in the presence of [^{3}H]NBMPR resulted in covalent labeling of a membrane protein(s) with a broad apparent M_r on SDS gel electropherograms of 77,000–45,000, similar to that previously reported for many other tissues, including human erythrocytes. We conclude that the maternal (brush-border) and fetal (basal) surfaces of the human placental syncytiotrophoblast possess broad-specificity, facilitated-diffusion, NBMPR-sensitive nucleoside transporters.

Key Words nucleoside transport · adenosine · nitrobenzylthioinosine (NBMPR) · microvillous and basal membrane vesicles · photoaffinity labeling · human placenta

Introduction

Nucleosides have diverse physiological and biological actions and as precursors of nucleotides can represent the building blocks of nucleic acids [44]. Adenosine modulates vascular tone, neural function, platelet aggregation and leucocyte activation [9]. Adenosine has recently been shown to be a vasoconstrictor in the human placenta vascular bed [38]. Most of these effects of adenosine are mediated by membrane-bound receptors and both adenosine A1 and A2 receptors have been characterized in human placental membranes [8, 36, 49]. It is therefore important to know the mechanism involved in controlling the concentration of extracellular adenosine since these processes may affect placental function and fetal development. To date, no study describing the properties of nucleoside transport by human placental plasma membrane have been presented.

Nucleosides cross mammalian cells by transport specific proteins, and the best studied of these systems is a facilitated-diffusion process that has a broad substrate specificity and is blocked by low concentrations (0.1–10 nM) of NBMPR [14, 35]. Inhibition by NBMPR is associated with tight, but reversible, binding of inhibitor to specific sites on the cell membrane [6, 17]. Upon exposure to UV light [^{3}H]NBMPR covalently binds to human erythrocyte band 4.5 polypeptides (apparent M_r 66,000–45,000) [19, 50]. A second facilitated-diffusion nucleoside transporter has been recognized that is insensitive to inhibition by NBMPR [3, 14, 18, 34, 35]. Some cell types exhibiting this system also lack high-affinity NBMPR binding sites although the majority of cultured cell lines usually coexpress NBMPR-sensitive and NBMPR-insensitive transporters [32, 34, 35]. Further heterogenicity in the transport of nucleosides is the finding that kidney and intestinal brush-border membrane vesicles

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transport nucleosides in a Na^+-dependent manner [15, 29, 30, 47]. To date, two nucleoside Na^+-cotransport systems that differ in their substrate specificity have been recognized [16]. In addition, K^+-dependent uridine transport by rat renal brush-border membrane vesicles has been demonstrated [27]. Interestingly, rabbit basolateral membrane vesicles from the renal outer cortex transport uridine by an NBMPR-sensitive facilitated-diffusion carrier [47].

Recently Wheeler and Yudilevich, using the isolated dually perfused guinea-pig placenta, demonstrated rapid transport and metabolism of adenosine at both maternal and fetal blood-tissue interfaces. However, this preparation did not allow the mechanism of cellular transport of nucleosides to be characterized due to extensive metabolism of adenosine by the placenta [46]. In an attempt to overcome this problem and given the interest and availability of human placenta, we have isolated human placental brush-border and basal membrane vesicles. Membrane vesicles, in addition, offer the advantage that the transport properties at each side of the placenta can be investigated independently of each other and the composition of the intravesicular and extravesicular fluid can be varied at will. In this paper the properties of adenosine transport and the binding of [^{3}H]NBMPR by human placental brush-border (maternal side) and basal (fetal side) membrane vesicles are described. Preliminary reports of part of this work have been presented [2, 12].

Materials and Methods

Preparation and Characterization of Membrane Vesicles

Syncytiotrophoblast basal and brush-border membrane vesicles from human placenta obtained within a few hours of delivery were prepared by established techniques [10, 23, 39]. The purity of the preparations was assessed by measuring the enrichment of the brush-border membrane enzyme, alkaline phosphatase [26], and the basal plasma marker, [^{3}H]dihydroalprenolol binding [23] as compared with the homogenate. Alkaline phosphatase activity in the brush-border membrane vesicles was on average enriched 15-fold compared to the homogenate. In contrast there was little contamination by alkaline phosphatase in basal membranes; enrichment factors ranged from 0.4- to 1.6-fold. [^{3}H]Dihydroalprenolol binding in basal membrane vesicles was more than 20-fold greater than binding in the homogenate. The membrane vesicles were resuspended in vesicle suspension medium (VSM; 300 mM mannitol, 5 mM HEPES-Tris, pH 7.4) and used within 24 hr of preparation for nucleoside uptake measurements. Freshly prepared membrane vesicles were revesiculated just before the nucleoside uptake assay by passing them 20 times through a 25-gauge syringe needle. In addition, aliquots of the membrane suspension were frozen and stored at −70°C until use.

In four preparations of brush-border and basal membrane vesicles, the intravesicular volume of the vesicles was determined using the apparent equilibrium spaces of two different nonmetabolized radioisotopes (3-O-methyl-[^{3}H]glucose and [^{3}H]uridine). The intravesicular volume for both isotopes was similar and the mean values (mean ± SEM(n)) were 1.16 ± 0.05(7) and 0.17 ± 0.03(8) μl/mg protein for brush-border and basal membrane vesicles, respectively.

Nucleoside Uptake by Membrane Vesicles

The uptake of [^{3}H]adenosine and [^{3}H]uridine (25 μCi/ml) at 22°C was measured by a rapid inhibitor-stop filtration technique [47] with the following modifications. The stop buffer used to terminate transport contained 10 μM dilazep in place of NBMPR or phlorhizin. In inhibition studies, test compounds and radiolabeled nucleoside were added simultaneously, except for NBMPR, dilazep and dipyridamole which were preincubated with the vesicles for 20 min. Blank values for transport were obtained by processing membrane samples exposed simultaneously to radioactively label nucleoside and 10 μM dilazep at 1°C. The initial rate of nucleoside uptake was estimated from analysis of the time course curves of uptake by fitting an exponential curve to the data using a nonlinear regression computer program (Enzfitter, Elsevier Biosoft). Initial zero-*trans* influx velocities were calculated according to the first derivative of the equation

$$v(t) = v_{eq}[1 - \exp(-kt)]$$

where k is the pseudo first-order rate constant and v and v_{eq} are the uptakes at various times and at equilibrium, respectively [*see* 33, 48].

Nucleoside Metabolism

Membrane vesicles were incubated with 33 μM [^{3}H]adenosine at 22°C and the reaction was terminated as described above. The filter was immediately shaken for 30 min at 22°C in 500 μl of 2 M NH_4OH [31]. The extract was lyophilized, resuspended in 30 μl of a standard solution containing adenosine, inosine, adenine, hypoxanthine and adenine nucleotides and 20 μl spotted on a silica-gel-coated plate containing a fluorescent indicator (Eastman, 0.1 mm thick). The solvent system was butan-1-ol : ethyl acetate : methanol : ammonia (7 : 4 : 3 : 4, vol/vol). After drying, the zones bearing the standards were localized under UV light (R_f values of 0, 0.19, 0.30, 0.49 and 0.75 adenine nucleotides, inosine, hypoxanthine, adenosine and adenine). The rest of the lane was equally divided into 1-cm individual zones. Each zone was cut from the plate, the strips were soaked in 0.5 ml of water to extract the radioactivity before addition of 4 ml of scintillation fluid (Optiphase RIA, LKB Scintillation Products). Uridine metabolism by the vesicles was also determined by TLC using the solvent systems described previously [47].

[^{3}H]NBMPR Binding Assay

Equilibrium [^{3}H]NBMPR binding to human placental membrane vesicles was performed as previously described for renal membrane vesicles using a total incubation volume of 3 ml [47]. Specific binding is defined as the difference between membrane content of [^{3}H]NBMPR in the presence and absence of 10 μM NBMPR.

Table. Metabolism of [^{3}H]adenosine and [^{3}H]uridine by human placental brush-border membrane vesicles

		Percent of ^{3}H recovered				
Nucleoside	Incubation (sec)	Adenosine/ uridine	Inosine	Hypoxanthine	Adenine/ uracil	Nucleotides
Adenosine (33 μM)	5	80	12	3	3	3
	60	51	32	1	6	6
Adenosine (33 μM) + 2'deoxycoformycin (100 μM)	5	91	2	3	3	0
	60	87	2	1	4	2
Uridine (33 μM)	60	95	—	—	2	0

Vesicles were incubated with [^{3}H]adenosine or [^{3}H]uridine (33 μM) for 5 and 60 sec at 22°C. Extraction and identification of the intravesicular products were performed as described in the text. Values are shown as a percentage distribution of total radioactivity detected on the TLC plate after subtraction of radioactivity bound to the filter in the absence of vesicles. Radioactivity that did not comigrate with one of the standards accounted for not more than 5% of the total ^{3}H recovered.

Photoaffinity Labeling with [^{3}H]NBMPR

Photoaffinity labeling of human placental brush-border and basal membrane vesicle suspensions and rabbit erythrocyte membranes with [^{3}H]NBMPR (8 nM) was performed under equilibrium binding conditions in the presence of 5 mM dithiothreitol as previously described for a variety of membrane preparations [19]. Treatment of the radiolabeled membrane preparations (5 mg/ml) with trypsin-TPCK (10 μg per mg protein) was carried out at 22°C in 50 mM sodium phosphate, pH 7.5, 1 mM EDTA and 100 mM NaCl for various times and stopped by the addition of 1 mM phenylmethylsulphonyl fluoride and an equal volume of SDS/polyacrylamide gel sample buffer [41]. Treatment with endoglycosidase-F (1 unit) was performed at 22°C for 18 hr in 100 mM sodium phosphate, pH 6.1, 75 mM β-mercaptoethanol, 50 mM EDTA, 0.5% (wt/vol) Triton X-100 and 0.05% (wt/vol) SDS. The enzymatic digestion was terminated by addition of an equal volume of SDS/polyacrylamide gel sample buffer [41]. Radioactivity associated with the membrane polypeptides was determined by SDS/polyacrylamide gel electrophoresis (10% acrylamide) and slicing the gel into 2-mm fractions as described previously [19].

Chemicals

[5,6-^{3}H]uridine (46 Ci/mmol), [2,5',8-^{3}H]adenosine (50 Ci/mmol), 1-[propyl-2,3-^{3}H]dihydroalprenolol (35 Ci/mmol) and [G-^{3}H]NBMPR (23 Ci/mmol) were obtained from Amersham, U.K. and Moravek Biochemicals, CA, respectively. Trypsin-TPCK treated, NBMPR, dipyridamole and polyethylenimine were purchased from Sigma. Endoglycosidase F was obtained from Boehringer Mannheim. Dilazep was a generous gift from Hoffman La Roche (Basel, Switzerland). All other reagents were of analytical grade.

Results

Metabolism of Nucleosides by Brush-Border Membrane Vesicles

In a previous study [46], using the guinea-pig perfused placenta, we had observed rapid metabolism of adenosine. The Table also demonstrates that human placental brush-border membrane vesicles metabolize adenosine principally to inosine. In contrast, no significant metabolism of uridine occurs within 60 sec of incubating brush-border membrane vesicles with [^{3}H]uridine (Table). Since metabolism can complicate the interpretation of uptake data we sought means of blocking adenosine metabolism. This was achieved using the potent and specific inhibitor of adenosine deaminase, 2'-deoxycoformycin at 100 μM [1]. Brush-border membrane vesicles preincubated with 2'-deoxycoformycin for 30 min exhibited no significant metabolism of adenosine. Therefore in subsequent kinetic experiments with adenosine, vesicles were preincubated with 100 μM 2'-deoxycoformycin. Previous studies have demonstrated that 2'-deoxycoformycin is a poor permeant of the nucleoside transporter in human erythrocytes (K_m > 10 mM) (*see* ref. [35]). Moreover, control studies demonstrated that the initial rate of 5 μM adenosine influx by placental membrane vesicles was similar in the presence and absence of 100 μM 2'-deoxycoformycin (0.37 ± 0.06 and 0.43 ± 0.04 pmol/mg protein/sec, respectively).

Time Course of Nucleoside Uptake by Brush-Border Membrane Vesicles

The time course of adenosine and uridine uptake (6.6 μM) by human placental brush-border membrane vesicles is shown in Fig. 1*A* and *B* in the presence of inwardly directed gradients of 100 mM NaCl or choline chloride. In contrast to renal and intestinal brush-border membrane vesicles [15, 16, 27, 29, 30], no marked stimulation of adenosine or uridine uptake by placental brush-border membrane vesicles was observed in the presence of a Na^+ electrochemical gradient (Fig. 1). The initial rate of adenosine influx at 6.6 μM, determined from the 0–60 sec time course (*see* Fig. 1*A*) as described in Materials and

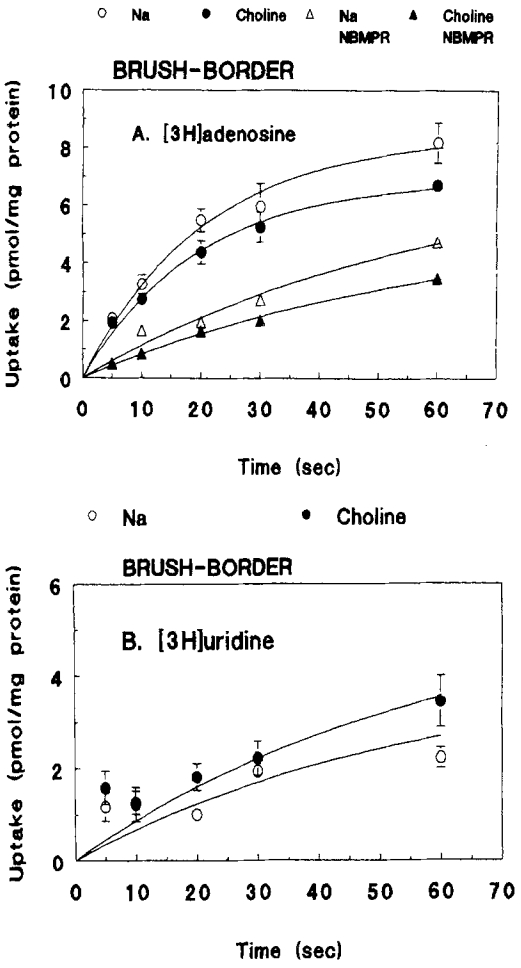

Fig. 1. Time course of adenosine and uridine uptake by human placental brush-border membrane vesicles. Vesicles were incubated with 6.6 μM [^{3}H]adenosine in the presence of 100 μM 2'-deoxycoformycin (*A*) or 6.6 μM [^{3}H]uridine (*B*) in the presence of inwardly directed gradients of 100 mM NaCl or 100 mM choline chloride. [^{3}H]adenosine uptake (*A*) was measured in the presence and absence of 10 μM NBMPR. Values are the mean ± SD of triplicate estimates

Methods, for three separate experiments was 0.60 ± 0.20 and 0.59 ± 0.18 pmol/mg protein/sec for NaCl and choline chloride, respectively (mean ± SEM). NBMPR (10 μM) was a potent inhibitor of adenosine uptake (Fig. 1*A*) and reduced the rate of influx to 30% of control: 1.03 ± 0.11(8) and 0.29 ± 0.05(7) pmol/mg protein/sec in the absence and presence of 10 μM NBMPR, respectively. The rate of NBMPR-sensitive uridine influx at 6.6 μM was 0.063 ± 0.011(3) which is 10-fold less than that of NBMPR-sensitive adenosine influx at the same concentration. Hence further studies were performed with the physiologically relevant nucleoside, adenosine.

In other experiments, the equilibrium value of uridine uptake was measured as a function of extravesicular osmolarity. The uptake of uridine was inversely proportional to the extravesicular osmolarity (213–340 mOsm) and extrapolation to infinite extravesicular osmolarity (zero intravesicular space) gave a uridine uptake value of 0.27 ± 0.35 pmol/mg protein (mean ± SD) that was not significantly different from zero. These results show that uptake of uridine occurred into an osmotically reactive intravesicular space and, furthermore, there is no significant binding of uridine to the membrane.

The effect of storage at −70°C for seven days on nucleoside uptake by the brush-border membrane vesicles was investigated. The membrane vesicles were revesiculated after thawing by passing them through a 25-gauge syringe needle. Both adenosine and uridine (6.6 μM) uptake by the vesicles was reduced after freezing. Initial rates of NBMPR-sensitive adenosine and uridine uptake from one placenta preparation were 0.39 and 0.09 pmol/mg protein/sec, respectively, for fresh vesicles and 0.08 and 0 pmol/mg protein/sec, respectively, for the same vesicle preparation stored at −70°C for seven days. Subsequent uptake studies used freshly isolated vesicles.

Kinetics of Adenosine Transport by Brush-Border Membrane Vesicles

The above results suggest that adenosine transport by brush-border membrane vesicles is mediated to a large extent by an NBMPR-sensitive facilitated-diffusion system. However, an NBMPR-insensitive facilitated-diffusion system has been found to be present in many animal cells in addition to the NBMPR-sensitive system (*see* Introduction). To test whether such an NBMPR-insensitive transport system was present in human placental brush-border membrane vesicles two approaches were used. First, the effect of a number of nucleoside transport inhibitors on adenosine influx was compared. Dilazep and dipyridamole at high concentrations (>1 μM) will inhibit the NBMPR-insensitive component of nucleoside transport [28]. Both compounds (10 μM) exhibited the same level of inhibition as NBMPR. Initial rates of adenosine influx (final conc. 33 μM) were 1.3, 0.40, 0.38 and 0.35 pmol/mg protein/sec for control, dipyridamole-, dilazep-, and NBMPR-treated vesicles. In the second approach the concentration dependence of adenosine influx in the presence and absence of 10 μM NBMPR was determined. Despite the low signal-to-noise ratio at high adenosine concentrations (radioactivity associated with membrane vesicles was only 30% higher than filter blank), adenosine uptake in the presence of 10 μM NBMPR was linear but in the absence of NBMPR uptake was best fitted to a linear plus

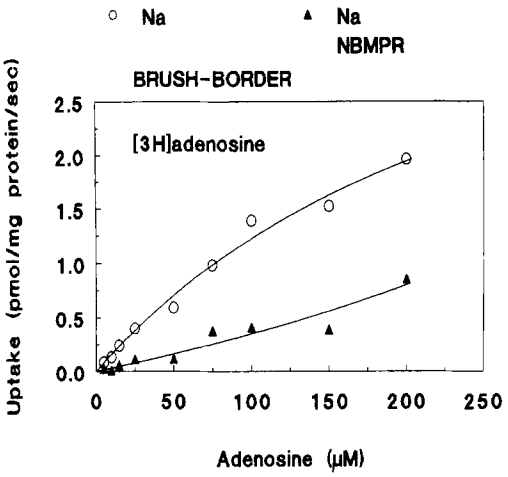

Fig. 2. Concentration dependence of adenosine influx by human placental brush-border membrane vesicles. Vesicles pretreated with 100 μM 2′-deoxycoformycin in the absence and presence of 10 μM NBMPR, were incubated with graded concentrations of [^{3}H]adenosine (0–200 μM final concentration) for 5 sec. NBMPR-sensitive adenosine uptake was taken, either as the rate of uptake in the absence of NBMPR minus the rate of uptake in the presence of NBMPR, or by fitting the total uptake rates to a two-component system, assuming a saturable and linear component. Nonlinear least-squares fit of the data gave similar kinetic constants for saturable NBMPR-sensitive adenosine influx with a K_m value of 142 ± 57 μM and a V_{max} of 1.9 ± 0.44 pmol/mg protein/sec

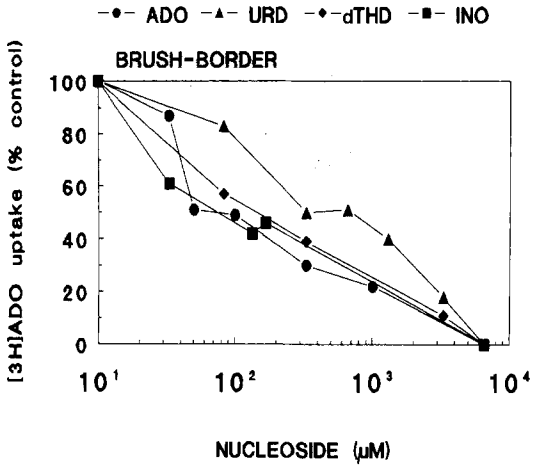

Fig. 3. Inhibition of adenosine uptake in human placental brush-border membrane vesicles by nucleosides. Initial rates of NBMPR-sensitive [^{3}H]adenosine uptake (6.6 μM) by vesicles in the presence of varying concentrations of nucleoside were determined from a complete time course for each concentration of inhibitor minus the initial rate of adenosine uptake in the presence of 10 μM NBMPR. Adenosine (ADO), uridine (URD), thymidine (dTHD) and inosine (INO). Values are expressed as a percentage of control initial adenosine uptake rates (total uptake less uptake in the presence of 10 μM NBMPR) and represent the pooled results of experiments conducted with five different membrane vesicle preparations. The average variation (SE) for each point was 15%

saturable component (Fig. 2). The kinetic constants of the saturable component shown in Fig. 2 were determined, either by the difference in uptake rates in the presence and absence of 10 μM NBMPR, or by fitting the total uptake data to a two-component system assuming a single Michaelis-Menten component and also a linear component. Least-squares fit of the data by both analysis procedures gave almost identical kinetic constants with an apparent K_m of 142 ± 57 μM and a V_{max} estimate of 1.9 ± 0.44 pmol/mg protein/sec. These values should only be regarded as an estimate because they are based on approximate initial rates (5-sec incubation only) and the high level of error introduced by the low signal. Nevertheless, the apparent K_m value for adenosine influx is similar to that observed for the NBMPR-sensitive nucleoside transporter in many other cell types [14, 16, 19, 35].

Inhibition of Adenosine Uptake by Nucleosides

The ability of other nucleosides to interact with the adenosine transport mechanism in human placental brush-border membrane vesicles was studied by determining the effect of various concentrations of a nucleoside (including self-inhibition) on the initial rate of adenosine influx (Fig. 3). It can be seen that inosine and thymidine appear to be as effective inhibitors as adenosine itself. An estimate of the IC_{50} (the concentration of nucleoside necessary to obtain 50% inhibition of the initial rate of adenosine influx) for these substrates appear to be about 100 μM. Uridine is the least effective of the nucleosides tested with an IC_{50} of approximately 600 μM. The nucleosides also reduced the rate of adenosine uptake to a similar value to that observed in the presence of NBMPR providing further evidence that the NBMPR-resistant component of adenosine uptake represents simple diffusion. The IC_{50} for adenosine self-inhibition is similar to the apparent K_m for adenosine uptake (Fig. 2).

[^{3}H] Adenosine Uptake by Basal Membrane Vesicles

Figure 4 shows the time course of 6.6 μM adenosine uptake by human placenta basal membrane vesicles in the presence and absence of 10 μM NBMPR. The uptake rates were low and no significant inhibition by NBMPR was observed (mean initial rates of adenosine uptake from four separate membrane preparations were 0.058 ± 0.017 and 0.065 ± 0.019 pmol/mg

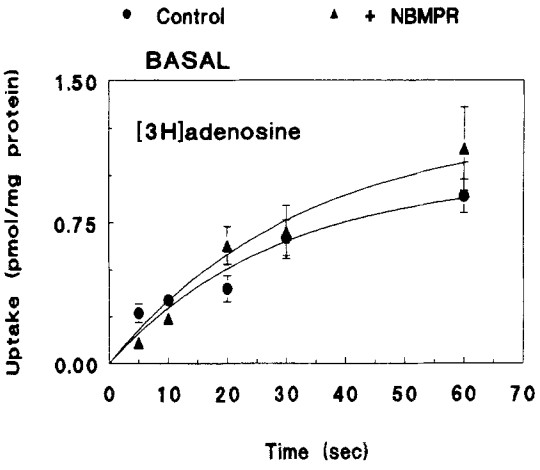

Fig. 4. Time course of adenosine uptake by human placental basal vesicles. Vesicles were incubated with 6.6 μM [^{3}H]adenosine in VSM medium in the presence (+NBMPR) or absence (control) of 10 μM NBMPR. Values are the means ±SD of triplicate estimates

protein/sec in the absence and presence of 10 μM NBMPR, respectively). These low uptake rates (on a mg protein basis) compared to those obtained with brush-border membrane vesicles (*see* Fig. 1) precluded the use of these basal membrane vesicles to characterize directly nucleoside transport. Interestingly, using these same membrane vesicles we were able to detect transport of other substrates such as 3-O-methyl glucose, alanine and choline (*data not shown, see also* ref. [5]).

[^{3}H]NBMPR Binding to Brush-Border and Basal Membranes

The concentration dependence of equilibrium [^{3}H]NBMPR binding to human placental brush-border and basal membrane are illustrated in Fig. 5 with the results of one experiment. [^{3}H]NBMPR binding was resolved into saturable and linear components. High concentrations of NBMPR (10 μM) eliminated saturable binding but had no effect on the linear component. Specific binding is defined as the difference in binding in the presence and absence of 10 μM NBMPR. From four brush-border and basal membrane preparations, respectively, the following parameters (mean ± SEM) were estimated by least-square analysis of the specific binding data using the computer program Enzfitter (Elsevier, Biosoft): apparent K_d 0.98 ± 0.21 and 1.05 ± 0.13 nM; B_{max} (maximal binding) 2.53 ± 0.63 and 1.60 ± 0.34 pmol/mg protein. Furthermore, Scatchard plots of the specific binding data were linear indicating a single population of high-affinity NBMPR binding sites in both membrane preparations.

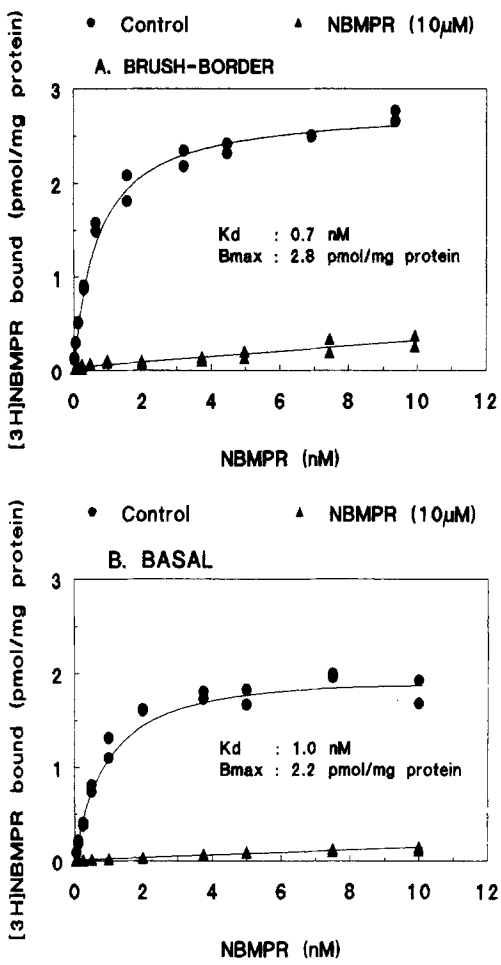

Fig. 5. Concentration dependence of [^{3}H]NBMPR binding by human placental brush-border (*A*) and basal (*B*) membrane vesicles. Membrane vesicles were incubated with graded concentrations of [^{3}H]NBMPR for 30 min at 22°C in the presence and absence (control) of 10 μM NBMPR. Membrane associated radioactivity is plotted against the final free concentration of [^{3}H]NBMPR. The individual data points of one experiment are shown

Inhibition of [^{3}H]NBMPR Binding by Nucleosides

Figure 6 shows the dose-response curves for inhibition of site-specific NBMPR binding by adenosine, inosine, uridine and thymidine. The apparent K_i values estimated from IC_{50} values for inhibition of NBMPR binding by brush-border and basal membrane vesicles were, respectively, 0.23 ± 0.012 and 0.14 ± 0.045 mM for adenosine, 0.36 ± 0.035 and 0.54 ± 0.046 mM for inosine, 0.70 ± 0.12 and 1.09 ± 0.18 mM for thymidine and 0.78 ± 0.10 and 1.26 ± 0.20 mM for uridine (mean ± SEM from three separate experiments). Moreover, adenosine in the presence of 2′-deoxycoformycin was shown to behave

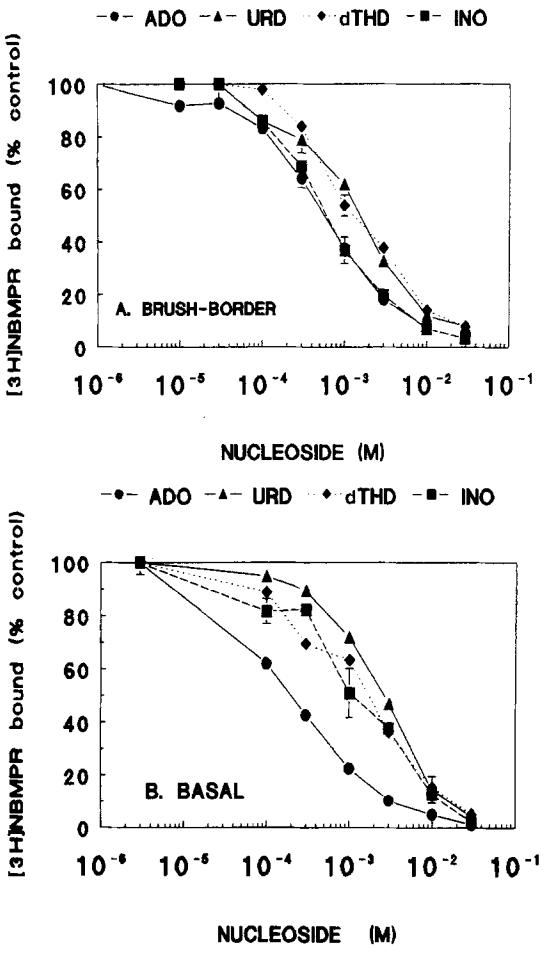

Fig. 6. Effect of nucleosides on site-specific [^{3}H]NBMPR binding to human placental brush-border (*A*) and basal (*B*) membrane vesicles. Site-specific (NBMPR-sensitive) [^{3}H]NBMPR binding (0.75 nM) was measured in the presence of adenosine (ADO), uridine (URD), thymidine (dTHD) and inosine (INO). Results are plotted as a percentage of control high-affinity [^{3}H]NBMPR binding activity in the absence of inhibitors (2.0 and 2.8 pmol/mg protein for brush-border and basal membranes, respectively). Values are means ±SD of triplicate estimates

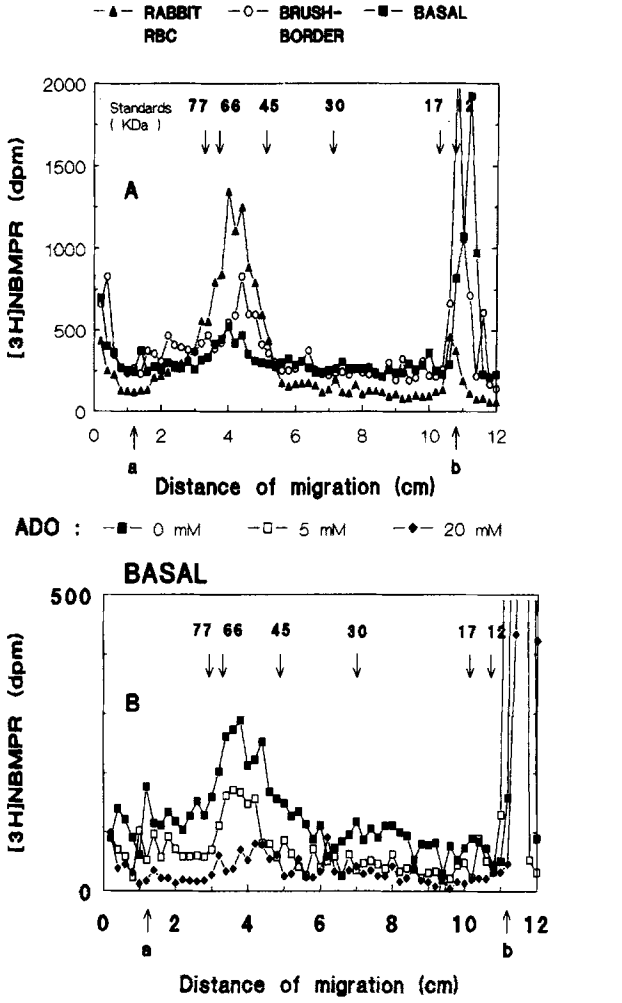

Fig. 7. Photoaffinity labeling of rabbit erythrocyte membranes and human placental brush-border and basal membranes with [^{3}H]NBMPR. (*A*) Membranes (rabbit erythrocyte, brush border and basal) equilibrated with 8 nM [^{3}H]NBMPR in the presence of 5 mM dithiothreitol were exposed to UV light for 30 sec at 4°C. Data for brush-border and basal placental membranes are multiplied by 1.5 for clarity. (*B*) Human placental basal membranes equilibrated with 8 nM [^{3}H]NBMPR in the absence and presence of 5 or 20 mM adenosine were supplemented with 5 mM dithiothreitol and exposed to UV light for 30 sec at 4°C. Samples were subjected to SDS/polyacrylamide gel electrophoresis as described in the text. Positions of the molecular weight standards are from the same slab gel. *a*, stacking gel-running gel interface; *b*, tracking dye front

as a competitive inhibitor of [^{3}H]NBMPR binding, apparent K_i values of 250 and 160 μM for brush-border and basal membrane vesicles, respectively. Dilazep and dipyridamole were also shown to be potent inhibitors of [^{3}H]NBMPR binding (IC_{50} values of 0.21 and 0.24 nM for dilazep and 7.5 ± 2.5(3) and 6.5 ± 0.9(3) nM for dipyridamole, for brush-border and basal membrane vesicles, respectively).

Photoaffinity Labeling with [^{3}H]NBMPR

Previous studies have described the use of NBMPR as a covalent photoaffinity probe of the NBMPR-sensitive nucleoside transporter in human erythrocytes and a variety of membrane preparations from tissues and cultured cells (*see* ref. [19] and references therein). Figure 7 compares the photoincorporation of [^{3}H]NBMPR into rabbit erythrocyte membranes and human placental brush-border and basal membrane vesicles. In all three cases, a major peak of radiolabeling was observed that comigrated within the apparent M_r region of 77,000–45,000. An additional lower radiolabeled molecular peak was

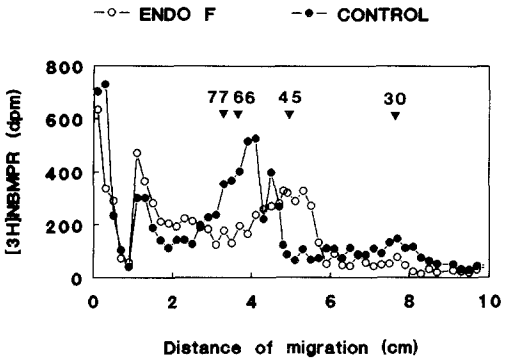

Fig. 8. Effect of endoglycosidase F on the electrophoretic mobility of [^{3}H]NBMPR photoaffinity labeled human placental brush-border membranes. Brush-border membranes photolabeled with [^{3}H]NBMPR were incubated for 18 hr at 22°C in the presence (endo F) and absence (control) of enzyme and electrophoresed as described in Materials and Methods. ^{3}H profiles and positions of M_r standards (kDa) are from the same slab gel

also observed in some membrane vesicle preparations (Figs. 7*B* and 8). This peak is likely to represent a degradation product of the nucleoside transporter [19, 20]. Previous results have shown that the NBMPR-labeled nucleoside transporter in rabbit erythrocytes comigrates with the human erythrocyte nucleoside transporter with a peak apparent M_r of 55,000 [20]. Covalent incorporation of [^{3}H]NBMPR into these polypeptides was abolished when photolysis was carried out in the presence of 10 μM NBMPR (*data not shown*) and 20 mM adenosine (Fig. 7*B*). In the same experiment 5 mM adenosine reduced [^{3}H] incorporation by about 50%. In other experiments, the effect of trypsin and endoglycosidase F digestion on the apparent M_r of the [^{3}H]NBMPR photolabeled proteins in brush-border membranes was investigated. Trypsin digestion (10 μg per mg protein for 15 min at 22°C) resulted in complete cleavage of the 45,000–77,000 radiolabeled protein, but in contrast to human erythrocytes no discrete smaller M_r fragments were resolved on the SDS-gel electropherogram [13]. Figure 8 shows that endoglycosidase F cleavage caused a shift of the ^{3}H peak to a lower apparent M_r of 46,000, a value similar to that previously obtained with the endoglycosidase F treated human erythrocyte nucleoside transporter [24]. The electrophoretic mobility of the presumed degradation ^{3}H peak was also shifted to a lower-M_r region of the gel, suggesting the polypeptide fragment(s) are also glycosylated.

Discussion

The human placental syncytiotrophoblast is a polarized epithelium with cytological similarities to intestinal and renal epithelium [43]. Extensive work comparing maternal and fetal side blood-tissue transport, and transplacental transfer, of various substrates, e.g., amino acids [51] and choline [40], has been made in the perfused guinea pig by Yudilevich and co-workers. In human placenta, even though there are many studies with brush-border vesicles, the work with basal vesicles is very scarce [*see* reviews 37, 51 and more recently 11]. This paper reports the first attempt to investigate the characteristics of nucleoside transport across the plasma membrane of both sides of the human trophoblast. It relates to our previous studies in the guinea pig in which adenosine [46] and uridine [45] transport were investigated. Evidence is provided which indicates that, in contrast to the polarized epithelia of kidney [47], brush-border and basal membrane vesicles isolated from the normal term human placentae, both possess an NBMPR-sensitive facilitated-diffusion system.

Brush-Border Membrane

Rapid metabolic degradation of adenosine (*see* the Table) has also previously been observed in the perfused guinea-pig placenta where metabolic transformation appeared to be due, in part, to an ectoenzyme [46]. In addition, an adenosine deaminase binding protein has been identified in human placenta [42] and isolated in some other tissues [4, 7]. Metabolism of adenosine by renal and intestinal brush-border membrane vesicles has also been demonstrated ([15]; T.C. Williams & S.M. Jarvis, *unpublished observations*), suggesting that in these tissues adenosine deaminase may either be membrane bound or become associated with the membrane during preparation of the vesicles. Absence of uridine metabolism by brush-border membrane vesicles (Table) was also observed in the guinea-pig placenta [45].

The saturable component of adenosine transport was independent of the sodium gradient having similar properties to the well-studied NBMPR-sensitive nucleoside transporter present in many other mammalian tissues including human erythrocytes [14, 19, 35]. Other purine and pyrimidine nucleosides, and the blockers dilazep and dipyridamole failed to inhibit adenosine influx further than that measured in the presence of NBMPR. This demonstrates that the linear component of adenosine uptake represents simple diffusion and not an NBMPR-insensitive facilitated-diffusion carrier (*see* Introduction). This high linear component could, in part, be an artifact inherent to the preparation of the vesicles. The rate of uridine influx was much lower than that of adenosine at the same extravesicular concentration and appeared, like adenosine, to be independent of the sodium gradient (Fig. 1). This again parallels the kinetic properties of the human erythrocyte nucleoside transporter [35].

Inhibition of adenosine transport by NBMPR was associated with high-affinity binding of the inhibitor to the brush-border plasma membrane with an apparent K_d similar to that reported for many other mammalian tissues [6, 14, 17, 35]. In addition, the nucleosides adenosine, inosine, thymidine and uridine, and the inhibitors dilazep and dipyridamole blocked [^{3}H]NBMPR binding with inhibition constants consistent with their relative affinity for NBMPR-sensitive nucleoside transporters in a variety of tissues [6, 14, 17, 19, 28, 35]. It should be noted that NBMPR binding to rat tissues is 10–100 times less sensitive to inhibition by dipyridamole and dilazep than other tissues [16, 18, 19].

Basal Membrane

[^{3}H]adenosine uptake by basal vesicles was less than 10% of that observed with brush-border membrane vesicles when the data is expressed on a mg protein basis (Fig. 3). However, basal vesicles are much smaller (*see* Materials and Methods) and when the uptake rates are expressed on the basis of intravesicular volume the difference in uptake rates between the two vesicle preparations was reduced to approximately twofold. Nevertheless, for basal vesicles it is difficult to interpret the [^{3}H]adenosine influx results due to the low vesicle uptake compared to the radioactivity associated with the filter blank. This may explain the apparent lack of inhibition of adenosine uptake by NBMPR despite the presence of high-affinity [^{3}H]NBMPR binding sites in the basal membrane preparation (Fig. 5*B*). An alternative explanation, but one that we do not favor, is that the NBMPR binding site has become "uncoupled" from the permeation site and thus NBMPR can no longer inhibit transport even though binding is not affected. It is also possible that some nucleoside transport activity is lost without an effect on [^{3}H]NBMPR binding during the isolation of the basal membrane vesicles as is observed after freezing. The properties (equilibrium binding constants and inhibition constants for nucleoside inhibition) of saturable high-affinity [^{3}H]NBMPR binding to basal membrane vesicles were similar to those observed for brush-border membrane vesicles (Figs. 5 and 6). Data on the study of [^{3}H]NBMPR binding sites in other epithelia is scarce. However, one of us has recently reported that rabbit renal outer cortical basolateral membranes possess specific NBMPR sites with equilibrium binding constants of 0.76 ± 0.46 nM for the apparent K_d with a B_{max} of 1.4 ± 0.9 pmol/mg protein [47]. These values are not significantly different from those measured in this study for basal and brush-border human placental membranes [47].

Photoaffinity Labeling and Molecular Properties of Placental Nucleoside Transporters

To the best of our knowledge, the present results represent only the second human tissue in which the transporter has been identified by photoaffinity labeling, the first being the human erythrocyte [50]. Our results strongly suggest that the labeled membrane polypeptides represent the NBMPR-sensitive nucleoside transporter. Preliminary studies using a polyclonal antibody against the human erythrocyte nucleoside transporter [25] detected a single polypeptide (M_r ~55,000) on Western blots for brush-border membrane vesicles (T. Davies, S.A. Baldwin, D.L. Yudilevich & S.M. Jarvis, *unpublished observations*). Interestingly, the antibody failed to detect any protein bands on Western blots performed with human placental basal membranes despite that fact that both membrane preparations had similar numbers of [^{3}H]NBMPR binding sites.

Possible Erythrocyte Contamination

The above studies strongly suggest that the nucleoside transporter systems in the human placenta share all the properties of the nucleoside transporter in human erythrocytes. This raises the possibility that the observations are in part due to erythrocyte contamination. An earlier study [44] has addressed this question of erythrocyte contamination by measuring the activity of the erythrocyte enzyme, acetylcholinesterase. The activity of acetylcholinesterase was 250- and 24-fold less in the brush-border and basal membrane vesicles compared with the erythrocyte membrane. Using the average number of [^{3}H]NBMPR binding sites of 30 pmol/mg of erythrocyte protein [17], the maximum level of [^{3}H]NBMPR binding derived from erythrocyte contamination would be 0.12 and 1.25 pmol/mg protein for brush-border and basal membrane vesicles, respectively. Thus, it is unlikely that the nucleoside transport activity in brush-border membrane vesicles arose from erythrocyte contamination, but for basal membrane vesicles this is a possibility. However, the above preliminary studies with an antibody to the human erythrocyte nucleoside carrier were unable to detect its activity on Western blots for basal membranes.

Conclusion

The present study has for the first time firmly demonstrated the presence of an NBMPR-sensitive facilitated-diffusion nucleoside transporter in brush-

border (maternal facing) membrane vesicles isolated from the human placenta. In addition, the results suggest that a similar nucleoside transport system may be present at the basal (fetal surface) of the syncytiotrophoblast of the human placenta. Glucose transport across the basal and microvillous membrane of the human placental syncytium also occurs by similar facilitated-diffusion transport mechanisms that are blocked by cytochalasin B [21, 22].

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References

1. Agarwal, R.P., Parks, R.E. 1977. Potent inhibition of 5-AMP-deaminase by nucleoside antibiotics coformycin and deoxycoformycin. *Biochem. Pharmacol.* **26**:663–666
2. Barros, L.F., Bustamante, J.C., Jarvis, S.M., Yudilevich, D.L. 1989. Binding of nitrobenzylthioinosine, an inhibitor of facilitated-diffusion nucleoside transport, to brush-border and basal membrane vesicles isolated from the human placenta. *J. Physiol. (London)* **418**:179P
3. Belt, J.A., Noel, L.D. 1985. Nucleoside transport in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells. Differences in sensitivity to nitrobenzylthioinosine and thiol reagents. *Biochem. J.* **232**:681–688
4. Bielat, K., Trisch, G.L. 1989. ECTO-enzyme activity of erythrocyte adenosine deaminase. *Mol. Cell. Biochem.* **86**:135–142
5. Bustamante, J.C., Jarvis, S.M., Yudilevich, D.L. 1990. Glucose and choline uptake and cytochalasin B binding in brush-border and basal membranes vesicles isolated from human placenta of normal and diabetic pregnancies. *J. Physiol. (London)* **423**:51P
6. Cass, C.E., Gaudette, L.A., Paterson, A.R.P. 1974. Mediated transport of nucleosides in human erythrocytes. Specific binding of the inhibitor nitrobenzylthioinosine to nucleoside transport sites in the erythrocyte membrane. *Biochim. Biophys. Acta* **345**:1–10
7. Daddona, P.E., Kelley, W.N. 1978. Human adenosine deaminase binding protein. Assay, purification and properties. *J. Biol. Chem.* **253**:4617–4623
8. Fox, I.H., Kurpis, L. 1983. Binding characteristics of an adenosine receptor in human placenta. *J. Biol. Chem.* **258**:6952–6955
9. Gerlach, E., Becker, B. 1987. Topics and Perspectives in Adenosine Research, Springer-Verlag, Berlin
10. Glazier, J.D., Jones, C.J.P., Sibley, C.P. 1988. Purification and uptake by human placental microvillus membrane vesicles prepared by three different methods. *Biochim. Biophys. Acta* **945**:127–134
11. Hoeltzli, S.D., Smith, C.H. 1989. Alanine transport systems in isolated basal plasma membrane of human placenta. *Am. J. Physiol.* **256**:C630–C637
12. Irribarra, V., Jarvis, S.M., Bustamante, J.C., Yudilevich, D.L. 1989. Adenosine transport by human placental brush-border membrane vesicles. *Biochem. Soc. Trans.* **17**:551–552
13. Janmohamed, N.S., Young, J.D., Jarvis, S.M. 1985. Proteolytic cleavage of [^{3}H]nitrobenzylthioinosine labelled nucleoside transporter in human erythrocytes. *Biochem. J.* **230**:777–784
14. Jarvis, S.M. 1988. Adenosine transporters. *Recept. Biochem. Methodol.* **11**:113–123
15. Jarvis, S.M. 1989. Characterisation of sodium-dependent nucleoside transport in rabbit intestinal brush-border membrane vesicles. *Biochim. Biophys. Acta* **979**:132–138
16. Jarvis, S.M. 1990. Chemical and molecular probes of nucleoside transport mechanisms in mammalian tissues. *In:* New Methods in the Study of Transport Across the Cell Membrane. D.L. Yudilevich, R. Devés, S. Perán, and Z.I. Cabantchick, editors. Plenum, New York *(in press)*
17. Jarvis, S.M., Young, J.D. 1980. Nucleoside transport in human and sheep erythrocytes. Evidence that nitrobenzylthioinosine binds specifically to functional nucleoside-transport sites. *Biochem. J.* **190**:377–383
18. Jarvis, S.M., Young, J.D. 1986. Nucleoside transport in rat erythrocytes: Two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and *p*-chloromercuriphenyl sulfonate. *J. Membrane Biol.* **93**:1–10
19. Jarvis, S.M., Young, J.D. 1987. Photoaffinity labelling of nucleoside transporter polypeptides. *Pharmacol. Ther.* **32**:339–359
20. Jarvis, S.M., Young, J.D., Wu, J.-S.R., Belt, J.A., Paterson, A.R.P. 1986. Photoaffinity labelling of the human erythrocyte glucose transporter with 8-azidoadenosine. *J. Biol. Chem.* **261**:11077–11085
21. Johnson, L.W., Smith, C.H. 1980. Monosaccharide transport across microvillous membrane of human placenta. *Am. J. Physiol.* **238**:C160–C168
22. Johnson, L.W., Smith, C.H. 1985. Glucose transport across the basal plasma membrane of human placental syncytiotrophoblast. *Biochim. Biophys. Acta* **815**:44–50
23. Kelley, L.K., Smith, C.H., King, B.F. 1983. Isolation and partial characterization of the basal cell membrane of human placental trophoblast. *Biochim. Biophys. Acta* **734**:91–98
24. Kwong, F.Y.P., Baldwin, S.A., Scudder, P.R., Jarvis, S.M., Choy, M.Y.M., Young, J.D. 1986. Erythrocyte nucleoside and sugar transport. Endo-β-galactosidase and endoglycosidase-F digestion of partially purified human and pig transporter proteins. *Biochem. J.* **240**:349–356
25. Kwong, F.Y.P., Davies, A., Tse, C.-M., Young, J.D., Henderson, P.J.F., Baldwin, S.A. 1988. Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography. *Biochem. J.* **245**:243–249
26. Lansing, A.I., Belkhode, M.L., Lynch, W.E., Lieberman, I. 1967. Enzymes of plasma membranes of liver. *J. Biol. Chem.* **242**:1772–1775
27. Lee, C.-W., Cheeseman, C.I., Jarvis, S.M. 1988. Sodium and potassium-dependent uridine transport in rat renal brush-border membrane vesicles. *Biochim. Biophys. Acta* **942**:139–149
28. Lee, C.-W., Jarvis, S.M. 1988. Kinetic and inhibitor specificity of adenosine transport in guinea-pig cerebral cortical synaptosomes: Evidence for two nucleoside transporters. *Neurochem. Int.* **12**:483–492
29. Le Hir, M., Dubach, U.C. 1985. Concentrative transport of purine nucleosides in brush border vesicles of the rat kidney. *Eur. J. Clin. Invest.* **15**:121–127

30. Le Hir, M., Dubach, U.C. 1985. Uphill transport of pyrimidine nucleosides in renal brush border vesicles. *Pfluegers Arch.* **404:**238–243
31. Li, C.C., Hochstadt, J. 1976. Membrane-associated enzymes involved in nucleoside processing by plasma membrane vesicles isolated from L929 cells grown in defined medium. *J. Biol. Chem.* **251:**1181–1187
32. Paterson, A.R.P., Jakobs, E.S., Harley, E.R., Cass, C.E., Robins, M.J. 1983. Inhibitors of nucleoside transport as probes and drugs. *In:* Development of Target-Orientated Anticancer Drugs. Y.C. Cheng, B. Gox, and M. Minkoff, editors. pp. 41–56, Raven, New York
33. Plagemann, P.G.W., Wohlhueter, R.M. 1980. Permeation of nucleosides, nucleic acid bases, and nucleotides in animal cells. *Curr. Top. Membr. Transp.* **14:**225–329
34. Plagemann, P.G.W., Wohlhueter, R.M. 1984. Nucleoside transport in cultured mammalian cells. Multiple forms with different sensitivity to inhibition by nitrobenzylthioinosine or hypoxanthine. *Biochim. Biophys. Acta* **773:**39–52
35. Plagemann, P.G.W., Wohlhueter, R.M., Woffendin, C. 1988. Nucleoside and nucleobase transport in animal cells. *Biochim. Biophys. Acta* **947:**405–443
36. Schocken, D.D., Schneider, M.N. 1986. Use of multiple radioligands to characterize adenosine receptors in human placenta. *Placenta* **7:**339–348
37. Shennan, D.B., Boyd, C.A.R. 1987. Ion transport by the placenta: A review of membrane transport systems. *Biochim. Biophys. Acta* **906:**437–457
38. Slegel, P., Kitagawa, H., Maguire, M.H. 1988. Determination of adenosine in fetal perfusates of human placental cotyledons using fluorescence derivatization and reverse-phase high-performance liquid chromatography. *Anal. Biochem.* **171:**124–134
39. Smith, N.C., Brush, M.G., Luckett, S. 1974. Preparation of human placental villous surface membrane. *Nature (London)* **252:**302–303
40. Sweiry, J.H., Yudilevich, D.L. 1985. Characterization of choline transport at maternal and fetal interfaces of the perfused guinea-pig placenta. *J. Physiol. (London)* **366:**251–266
41. Thompson, S., Maddy, A.H. 1982. Gel electrophoresis of erythrocyte membrane proteins. *In:* Red Cell Membranes—A Methodological Approach. J.C. Ellory and J.D. Young, editors. pp. 67–94. Academic, London
42. Trotta, P.P. 1982. Identification of a membrane adenosine deaminase binding protein from human placenta. *Biochemistry* **21:**4014–4023
43. Truman, P., Ford, H.C. 1984. The brush border of the human term placenta. *Biochim. Biophys. Acta* **779:**139–160
44. Vanderpuye, O.A., Kelley, L.K., Morrison, M.M., Smith, C.H. 1988. The apical and basal plasma membranes of the human placental syncytiotrophoblast contain different erythrocyte membrane protein isoforms. Evidence for placental forms of band 3 and spectrin. *Biochim. Biophys. Acta* **943:**277–287
45. Wheeler, C.P.D., Yudilevich, D.L. 1987. Uptake of uridine and its inhibition by nucleoside transport inhibitors in the perfused guinea-pig placenta. *J. Physiol. (London)* **396:**40P
46. Wheeler, C.P.D., Yudilevich, D.L. 1988. Transport and metabolism of adenosine in the perfused guinea-pig placenta. *J. Physiol. (London)* **405:**511–526
47. Williams, T.C., Doherty, A.J., Griffith, D.A., Jarvis, S.M. 1989. Characterization of sodium-dependent and sodium-independent nucleoside transport systems in rabbit brush-border and basolateral plasma-membrane vesicles from the renal outer cortex. *Biochem. J.* **264:**223–231
48. Wohlhueter, R.M., Marz, R., Graff, J.C., Plagemann, P.G.W. 1978. A rapid-mixing technique to measure transport in suspended animal cells: Applications to nucleoside transport in Novikoff rat hepatoma cells. *Methods Cell. Biol.* **20:**211–236
49. Work, C., Hutchinson, K., Prasad, M., Bruns, R.F., Fox, I.H. 1989. Characteristics of an adenosine A_1 binding site in human placental membranes. *Arch. Biochem. Biophys.* **268:**191–202
50. Wu, J.-S.R., Kwong, F.Y.P., Jarvis, S.M., Young, J.D. 1983. Identification of the erythrocyte nucleoside transporter as a band 4.5 polypeptide. Photoaffinity labelling studies using nitrobenzylthioinosine. *J. Biol. Chem.* **258:**13745–13751
51. Yudilevich, D.L., Sweiry, J.H. 1985. Transport of amino acids in the placenta. *Biochim. Biophys. Acta* **822:**169–201

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