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Nucleoside transport in cultured LLC-PK epithelia

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The transport of nucleosides by LLC-PK$_1$ cells, a continuous epithelial cell line derived from pig kidney, was characterised. Uridine influx was saturable (apparent $K_m \sim 34$ μM at 22°C) and inhibited by > 95% by nitrobenzylthioinosine (NBMPR), dilazep and a variety of purine and pyrimidine nucleosides. In contrast to other cultured animal cells, the NBMPR-sensitive nucleoside transporter in LLC-PK$_1$ cells exhibited both a high affinity for cytidine (apparent $K_i \sim 65$ μM for influx) and differential 'mobility' of the carrier (the kinetic parameters of equilibrium exchange of formycin B are greater than those for formycin B influx). An additional minor component of sodium-dependent uridine influx in LLC-PK$_1$ cells became detectable when the NBMPR-sensitive nucleoside transporter was blocked by the presence of 10 μM NBMPR. This active transport system was inhibited by adenosine, inosine and guanosine but thymidine and cytidine were without effect, inhibition properties identical to the N1 sodium-dependent nucleoside carrier in bovine renal outer cortical brush-border membrane vesicles (Williams and Jarvis (1991) Biochem. J. 274, 27–33). Late proximal tubule brush-border membrane vesicles of porcine kidney were shown to have a much reduced Na^+-dependent uridine uptake activity compared to early proximal tubule porcine brush-border membrane vesicles. These results, together with the recent suggestion of the late proximal tubular origin of LLC-PK$_1$ cells, suggest that in vivo nucleoside transport across the late proximal tubule cell may proceed mainly via a facilitated-diffusion process.

Introduction

Mammalian cells have been shown to possess a number of carrier systems mediating nucleoside transport [1,2]. The existence of two equilibrative facilitated-diffusion transporters that accept both purine and pyrimidine nucleosides as permeants has been established. These transporters have similar substrate specificity and kinetic properties but can be distinguished by differences in their sensitivity to inhibition by the nucleoside analog nitrobenzylthioinosine (NBMPR) [3–5]. In addition to these facilitated-diffusion systems, measurements of the renal clearance of adenosine in humans and mice suggested the existence of a concentrative mechanism in the kidney responsible for the reabsorption of filtered adenosine [6]. More recently, studies using brush-border membrane vesicles prepared from the proximal tubule of rat, rabbit and bovine kidney have confirmed the presence of active nucleoside transport in the kidney by demonstrating the presence of two Na^+-coupled transport systems for nucleosides [7–14]. One system designated N1, accepts purine nucleosides and uridine whereas the other system, N2, has a substrate specificity for pyrimidine nucleosides, adenosine and analogues of adenosine. These Na^+-dependent systems are resistant to inhibition by NBMPR [8,12,14].

A limitation in the use of membrane vesicles is that regulation of transport is difficult to study since the transport mechanism of interest is often severed from intracellular regulatory events. Furthermore, there is still a need to make direct measurements of nucleoside transport at both the apical and serosal membrane surfaces of intact cells. One approach to study both epithelial transport and its regulation is to use cultured epithelia. LLC-PK$_1$ is a continuous renal epithelial cell line derived from the juvenile pig kidney [15] that has been widely used as a model for the proximal tubular epithelium. LLC-PK$_1$ cells not only retain the typical morphological properties of transporting epithelia in vitro, but also express high levels of proximal brush-

Abbreviations: NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid (disodium salt); IC_{50}, concentrations causing half-maximal inhibition; NMG^+, *N*-methyl-D-glucamine.

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border membrane enzymes and a number of transport systems characteristic of the renal proximal tubule [16].

The objective of this study was to characterize nucleoside transport in LLC-PK_1 cells in the hope that it would provide an intact cell system in which the properties and regulation of Na^+-dependent nucleoside transport could be investigated. Surprisingly, we were only able to detect a minor component (less than 5% of the total flux) of Na^+-dependent nucleoside transport in LLC-PK_1 cells under our cell culture conditions. Our results, however, demonstrate that LLC-PK_1 cells possess an NBMPR-sensitive facilitated-diffusion nucleoside transporter with properties that differ from those in other cultured cell lines studied.

Materials and Methods

Cell culture

LLC-PK_1 cells were obtained from the American Type Culture Collection at passage 199 and were used between passages 212 and 235. The cells were passaged in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 25 mM Hepes (pH 7.4), 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated fetal calf serum. For experiments, except where otherwise stated, 2 cm^2 wells on a 24 well plate were seeded with $2 \cdot 10^5$ cells in 1 ml of medium. In addition, this medium also contained 25 ng/ml amphotericin B to guard against possible fungal contamination in these relatively exposed plates. The cells were fed with fresh medium every 2 days. Cells used in the present study were free of mycoplasma as detected using Hoechst 33258 stain.

Transport studies

Nucleoside influx was assayed at room temperature (22–24°C). The culture medium was aspirated and wells containing cell monolayers were rinsed three times with 1.0-ml aliquots of a medium containing (in mM): 145 KCl, 4.2 $KHCO_3$, 0.36 $K_2HPO_4 \cdot 3H_2O$, 1.3 $CaCl_2 \cdot 2H_2O$, 0.44 KH_2PO_4, 0.5 $MgCl_2 \cdot 6H_2O$ and 10 Hepes (pH 7.4) [17]. Uptake was initiated by the addition of 0.2 ml of the above medium with the following substitutions: 140 mM NaCl, 4.2 mM $NaHCO_3$ and 5 mM KCl substituted for 145 mM KCl and 4.2 mM $KHCO_3$, for Na^+-dependent transport or 140 mM *N*-methyl-D-glucamine (HCl) and 5 mM KCl in place of 145 mM KCl for Na^+-independent transport. The transport medium also contained [^{3}H]nucleoside (10 μCi/ml), inulin[^{14}C]carboxylic acid (0.4 μCi/ml) and further additions as indicated in the legends. After the appropriate time interval, transport was terminated by aspirating the medium and washing the monolayer three times with 1.0-ml aliquots of ice-cold Na^+-free medium (total time, 10 s). Cells were lysed by the addition of 0.2 ml of 0.5 M NaOH and assayed for radioactivity. In inhibition studies, test compounds and [^{3}H]uridine were added to cell monolayers simultaneously, except for NBMPR and dilazep which were preincubated with the cells for 10 min before addition of [^{3}H]uridine.

In some experiments, the equilibrium exchange influx of formycin B, a non-metabolized nucleoside [18] was measured. Varying concentrations of formycin B (0–10 mM) were added to each culture well and incubated at 37°C for 60 min. The wells were then rapidly washed and the inward flux of [^{3}H]formycin B at the same concentration as the preincubation mixture determined as described above for nucleoside influx.

The results of uptake experiments, corrected for adhering extracellular medium using inulin[^{14}C]carboxylic acid, an extracellular marker, are expressed per milligram of protein as determined by the method of Lowry et al. [19]. Intracellular volume was determined from the distribution ratio at equilibrium (1–2 h) of L-[^{3}H]alanine or 3-*O*-methyl-D-[1-^{3}H]glucose, uptake being measured as described above for ^{3}H-labelled nucleosides in the absence of Na^+.

Uridine metabolism

Transport was assayed as described above except that inulin[^{14}C]carboxylic acid was omitted. Immediately following termination of uptake, monolayers were covered with 200 μl of ice-cold 4% perchloric acid. The acid extracts were further processed and analyzed by TLC using PK5F silica gel preparative TLC plates impregnated with fluorescent indicator (Whatman, 1 mm) as described previously [13,20].

[^{3}H]NBMPR binding

Equilibrium [^{3}H]NBMPR binding measurements to LLC-PK_1 cells were performed on cells in suspension harvested from subconfluent monolayers. [^{3}H]NBMPR binding was determined by the oil stop method that involves separating the associated [^{3}H]NBMPR with the cells from free [^{3}H]NBMPR by centrifugation through an oil layer (Dow Corning 550: 220/1, 80:20) as described previously [21].

Preparation of cortical and medulla membrane vesicles and nucleoside uptake

Porcine renal cortical and medulla brush-border membrane vesicles were prepared from the outer regions of fresh pig kidney cortex and medulla, using a $MgCl_2$-precipitation method [11,14]. The uptake of ^{3}H-labelled nucleosides (20 μCi/ml) at 22°C into the brush-border membrane vesicles was measured with a phloridizin stop-filtration method as detailed previously for rat and bovine vesicles [11,14].

Data analysis

All transport and binding experiments were carried out in triplicate unless otherwise stated. The errors

given in the tables are standard errors of the mean and those in the figures are standard deviations. In least-squares fits to the data, points were weighted according to the inverse of their relative experimental errors.

Materials

Cell culture reagents and plasticware were purchased from Gibco, Paisley, UK and Flow Laboratories, Glasgow, UK. [5,6-^{3}H]Uridine (46 Ci/mmol), L-[2,3-^{3}H]alanine (47 Ci/mmol), [6-^{3}H]thymidine (25 Ci/mmol), 3-*O*-methyl-D-[1-^{3}H]glucose (2.7 Ci/mmol) and inulin[^{14}C]carboxylic acid (10.7 mCi/mmol) were obtained from Amersham International (Amersham, UK). [G-^{3}H]Formycin B (7 Ci/mmol), [G-^{3}H]NBMPR (23 Ci/mmol) and [8-^{3}H]guanosine (5 Ci/mmol) were purchased from Moravek Radiochemicals (Brea, CA, USA) and ICN Radiochemicals (Irvine, CA, USA), respectively. Dilazep was a generous gift from Hoffman La Roche (Basal, Switzerland). NBMPR, ouabain, amphotericin B and monensin were obtained from Sigma Chemical Co (Poole, Dorset, UK). All other reagents were of analytical grade.

Results

Uridine uptake and metabolism by LLC-PK$_1$ cells

To determine conditions where initial rates of uridine transport could be measured, the uptake and metabolism of 100 and 5 μM uridine were examined. Uridine uptake at both concentrations was linear for up to 2 min (see Fig. 1). Analysis of the intracellular tritium in LLC-PK$_1$ cells by TLC revealed that at an extracellular concentration of 5 μM uridine was rapidly phosphorylated to nucleotides (85% following an incubation period of 1 min). In contrast, at 100 μM extracellular uridine, 79% of the intracellular radioactivity at 1 min comigrated with uridine. The intracellular aqueous volume, measured from the apparent equilibrium space using the non-metabolised radioisotope, L-[^{3}H]alanine (see Materials and Methods), was estimated to be 4.71 ± 0.26 μl/mg protein. Calculations based on this value demonstrated that the intracellular uridine concentration at 1 min was < 20% of that in the extracellular medium at both 5 and 100 μM, and as such would not result in significant backflux thereby effecting the initial rate measurements [1]. This result, together with the linearity of the time course for uridine uptake, demonstrates that initial rates of uridine transport by LLC-PK$_1$ cells can be estimated using a 60-s time interval.

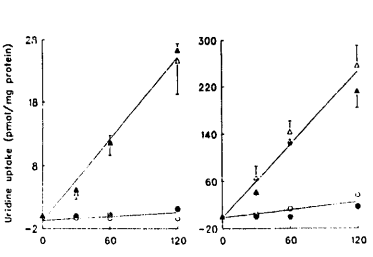

Fig. 1. Time course of uridine uptake by 3-day-old confluent LLC-PK$_1$ monolayers. The uptake of uridine (5 μM, left panel; 100 μM, right panel) at 22°C was determined at the indicated times in the presence of 140 mM NaCl in the absence (▲) or presence (●) of 10 μM NBMPR, and in 140 mM NMG$^+$ in the absence (△) or presence (○) of 10 μM NBMPR.

Fig. 1 also shows the time course of uridine uptake (5 μM) by confluent monolayers (3-day-old) of LLC-PK$_1$ cells in the presence and absence of a Na^+ electrochemical gradient. An inwardly directed Na^+ electrochemical gradient (140 mM outside) had no apparent effect on the initial rate of uridine influx in LLC-PK$_1$ cells as compared to the flux in the presence of NMG^+. Furthermore, the uptake of uridine was inhibited (> 95%) by 10 μM NBMPR, a potent inhibitor of facilitated-diffusion nucleoside transport in many cultured cells [1,2]. Control experiments established that our batch of LLC-PK$_1$ cells exhibited Na^+-dependent alanine uptake (2.34 ± 0.22 and 0.58 ± 0.18 nmol/mg protein per min in the presence and absence of Na^+, respectively; 250 μM alanine) confirming the results of earlier studies [17].

It has recently been reported that in a variety of mouse cells lines [22] Na^+-dependent concentrative nucleoside transport becomes apparent only when the cells are incubated for prolonged time periods in the presence of an inhibitor of equilibrative transport, such as NBMPR. The results of such an experiment are shown in Fig. 2. Under these circumstances a minor component of uridine uptake that was dependent on Na^+ was revealed. At an extracellular concentration of 5 μM uridine, the Na^+-dependent component of influx represented only 3% of the total initial flux (7.5 ± 0.8 pmol/mg protein per min). The rate of Na^+ stimulated uridine transport was independent of the age of the monolayer (data not shown).

The above results suggest that LLC-PK$_1$ cells exhibit both NBMPR-sensitive and Na^+-dependent uridine transport. However, the magnitude of the latter component is so low as to be hardly detectable. In subsequent transport studies with LLC-PK$_1$ monolayers, NMG^+ transport media was used to explore the characteristics of the Na^+-independent transport route whereas the difference in transport rates between Na^+ and NMG^+ media in the presence of NBMPR defined the Na^+-dependent transport component.

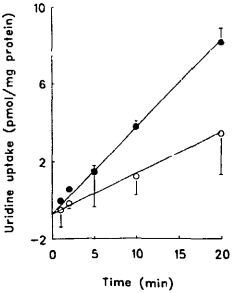

Fig. 2. Prolonged time course of uridine uptake by 3-day-old confluent LLC-PK$_1$ monolayers. LLC-PK$_1$ monolayers were preincubated with 10 μM NBMPR and the uptake of 5 μM uridine at 22°C determined in the presence of 140 mM NaCl (●) or 140 mM NMG$^+$ (○).

Uridine transport by porcine renal brush-border membrane vesicles

Although Na^+-dependent nucleoside transport had been demonstrated in brush-border membrane vesicles from the outer cortex of rat, rabbit and bovine kidneys [7–14], no such studies with porcine cortical brush-border membrane vesicles had been conducted. If such vesicles lacked substantial expression of Na^+-dependent nucleoside transport this would possibly explain the virtual absence of Na^+-coupled nucleoside transport in LLC-PK$_1$ cells. However, the data in Fig. 3 demonstrates Na^+-dependent uridine uptake in porcine outer cortical renal brush-border membrane vesicles. The initial rate of uridine influx was stimulated 4-fold by an inwardly directed gradient of 100 mM NaCl compared to choline chloride. Nevertheless, the initial rate of 5 μM Na^+-dependent uridine influx was approximately 40, 10 and 2-fold less than that observed with rat, bovine and rabbit outer cortical brush-border membrane vesicles, respectively [11–14]. Interestingly, uridine transport in brush-border membrane vesicles isolated from the outer medulla of pig kidney was only slightly stimulated by the presence of an inwardly directed 100 mM NaCl gradient compared to a choline chloride gradient (Fig. 3). The Na^+-dependent component of uridine influx (uptake in the presence of NaCl minus that in the presence of choline chloride) by porcine renal medulla brush-border membrane vesicles was 4-fold less than that observed with renal cortical membrane vesicles (0.15 ± 0.03 versus 0.55 ± 0.09 pmol/mg protein per s). Moreover, no apparent overshoot in the uptake of uridine was observed in renal medulla vesicles. However, the final equilibrium value for uridine uptake, measured at 60 min, was similar for both renal medulla and cortical vesicles (Fig. 3). The possibility that the Na^+-dependent component of nucleoside transport in porcine renal medulla brush-border membrane vesicles was due to contaminating cortical membrane vesicles cannot be ruled out but seems unlikely in view of the ease by which the two regions of the kidney can be discriminated in pig.

Concentration dependence of Na^+-independent uridine transport

The initial rate of uridine influx by LLC-PK$_1$ cells in NMG^+ transport media as a function of the external uridine concentration in the range 5 to 300 μM was determined. Total uptake was the sum of saturable and

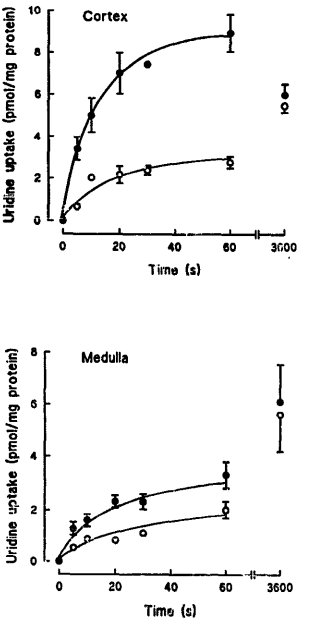

Fig. 3. Time course of uridine uptake by porcine renal brush-border membrane vesicles. Brush-border membrane vesicles (10 μl) from the outer regions of the porcine kidney cortex (upper panel) and medulla (lower panel) were incubated at 22°C with 20 μl of media containing (final concn.) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and either 100 mM NaCl (●) or 100 mM choline chloride (○).

nonsaturable processes. The saturable component, which conformed to simple Michaelis-Menten kinetics, was obtained by subtracting the linear component estimated in the presence of 10 μM NBMPR (obtained by linear regression) from the total uptake values and is shown in Fig. 4. Nonlinear regression analysis using the computer program Enzfitter (Elsevier Biosoft) of the resultant data yielded apparent K_m and V_{max} values for the NBMPR-sensitive saturable component of 41 ± 7 μM and 2.5 ± 0.14 pmol/mg protein per s, respectively. A similar experiment with a different batch of cells gave values of 27 ± 7 μM and 1.3 ± 0.12 pmol/mg protein per s for the K_m and V_{max}, respectively.

Inhibition of Na^+-independent uridine transport

The NBMPR-sensitivity and substrate specificity of the uridine transport mechanism was studied by investigating the effect of NBMPR, dilazep and nucleosides on the influx of uridine by LLC-PK_1 cells. The results are presented in Table I. NBMPR and dilazep inhibited uridine influx with apparent K_i values of 2.7 ± 0.75 and 0.83 ± 0.25 nM (mean ± S.E. (n = 3); estimated from the IC_{50} value). Inclusion of eserine (10 μM), an ester hydrolase inhibitor, had no significant effect on the potency of dilazep inhibition (apparent K_i 0.69 ± 0.16 nM). Of the nucleosides tested, adenosine was the most effective inhibitor, followed by thymidine, cytidine, guanosine, inosine and formycin B, with apparent K_i values of 25 ± 4, 31 ± 7, 64 ± 12, 68 ± 31, 106 ± 45 and 118 ± 11 μM, respectively. This result is in agreement with the known relative affinities of these nucleosides for NBMPR-sensitive transport [1,2], with the exception of cytidine which has been reported to exhibit a lower affinity (K_m of 2–4 mM) [23] than we observed in the present study. Similar K_i values for cytidine inhibition of 100 μM [^{3}H]uridine influx were also obtained (K_i values of 81 and 73 μM for two separate experiments). Nucleoside inhibition profiles were monophasic and parallel, suggesting competitive inhibition of a single class of nucleoside transporters.

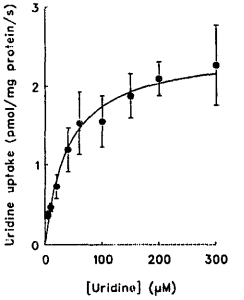

Fig. 4. Concentration dependence of uridine influx in LLC-PK_1 monolayers. LLC-PK_1 monolayers (3-day-old) were incubated for 1 min at 22°C with NMG$^+$ and graded concentrations of [^{3}H]uridine in the presence or absence of 10 μM NBMPR. The difference between cell associated ^{3}H in the presence and absence of NBMPR was plotted. The kinetic constants were determined by non-linear least-squares fit of the Michaelis Menten equation and gave a K_m value of 41 ± 7 μM with a V_{max} of 2.5 ± 0.14 pmol/mg protein per s.

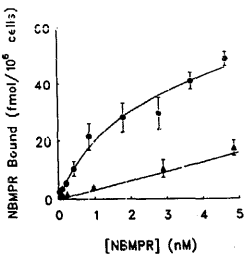

Fig. 5. Concentration dependence of [^{3}H]NBMPR binding to suspended LLC-PK_1 cells. Suspended LLC-PK_1 cells were incubated with graded concentrations of [^{3}H]NBMPR for 30 min at 22°C in the presence (▲) or absence (○) of 10 μM NBMPR. Specific binding of [^{3}H]NBMPR represents the difference between membrane associated NBMPR in the presence and absence of NBMPR and was saturable (K_d = 0.92 ± 0.33 nM; B_{max} = 33 ± 4 fmol/10^6 cells).

[^{3}H]NBMPR binding

The association between high-affinity NBMPR binding sites and functional nucleoside carriers in cells in which transport is inhibited by NBMPR is now well established [1,2]. Thus, [^{3}H]NBMPR was employed as a specific binding probe enabling further characterisation of the nucleoside transporter in suspended LLC-PK_1 cells. Fig. 5 illustrates the results of an experiment in which specific [^{3}H]NBMPR binding, defined as the difference in the amount bound in the presence and absence of 10 μM unlabelled NBMPR, was saturable with an apparent K_d of 0.92 ± 0.33 nM and a B_{max} (maximal binding) of 33 ± 4 fmol/10^6 cells. The mean values from three experiments were 0.80 ± 0.18 nM for the apparent K_d with a B_{max} of 36 ± 7.2 fmol/10^6 cells (mean ± S.E.). Hill coefficients calculated from the saturable binding data did not significantly differ from 1, indicating the presence of a single class of high-affinity NBMPR binding sites. The K_i value for NBMPR inhibition of uridine transport is similar to the apparent K_d value for NBMPR-binding. Calculations based on the B_{max} value presented, estimate the total number of binding sites per cell at (0.21 ± 0.04) · 10^5. Assuming that each NBMPR binding site repre-

sents a single transporter the turnover number for NBMPR-sensitive uridine influx is estimated to be 10 ± 2 molecules/site per s at room temperature assuming a V_{max} of 2.5 ± 0.14 pmol/s per mg of protein.

The effect of a variety of nucleosides on the site-specific binding of [^{3}H]NBMPR was explored and the results are summarized in Table I. Once again adenosine was the most effective of the nucleosides tested with apparent K_i values 91, 170, 205, 250, 455 and 760 for adenosine, thymidine, inosine, uridine, formycin B and cytidine, respectively (means of two separate experiments).

Equilibrium exchange influx of formycin B

The K_i values for inhibition of specific [^{3}H]NBMPR binding were between 2- and 12-fold higher than the K_i values for inhibition of uridine influx (see Table I). Previous results with mammalian erythrocytes have shown that such a discrepancy is eliminated when the K_m values for equilibrium exchange are used instead of the K_m or K_i values for influx [2,24,25]. Thus, using the non-metabolised nucleoside, formycin B [18], we compared the kinetic properties of formycin B influx under zero-*trans* and equilibrium exchange conditions

TABLE I

Effect of nucleoside and transport inhibitors on Na$^+$-independent uridine influx and specific NBMPR binding by LLC-PK$_1$ cells

The initial rate of uridine influx at 22°C was determined by the addition of 10 μM [^{3}H]uridine in NMG$^+$ medium and test compound to LLC-PK$_1$ monolayers. For dilazep and NBMPR, LLC-PK$_1$ monolayers were preincubated with these compounds as detailed in Materials and Methods. K_i values were calculated from the equation $K_i = IC_{50}/(1 + L/K_m)$, where the K_m value was taken as 34 μM and $L = 10$ μM uridine. The values are the means ± S.E. of at least three separate experiments. [^{3}H]NBMPR binding was initiated by the addition of 1 nM [^{3}H]NBMPR in the presence or absence of nucleosides to suspended LLC-PK$_1$ cells treated with or without 10 μM NBMPR. After 30 min at 22°C, [^{3}H]NBMPR bound to cells was determined as described in Materials and Methods. Specific [^{3}H]NBMPR bound was calculated as the difference between total binding and binding in the presence of 10 μM NBMPR. K_i values were calculated from the equations $K_i \approx IC_{50}/(1 + L/K_d)$, where the K_d value was taken as 0.80 nM. Individual values from two separate experiments are given. n.d., not determined.

Inhibitor	Apparent K_i value	
	uridine influx	[^{3}H]NBMPR binding
NBMPR	2.7 ± 0.75 nM	n.d.
Dilazep	0.83 ± 0.25 nM	n.d.
Dilazep + eserine (10 μM)	0.69 ± 0.16 nM	n.d.
Adenosine	25 ± 4 μM	62, 120 μM
Inosine	106 ± 45 μM	240, 170 μM
Formycin B	118 ± 11 μM	360, 550 μM
Thymidine	31 ± 7 μM	120, 220 μM
Cytidine	64 ± 12 μM	760, 760 μM
Uridine	n.d.	275, 225 μM
Guanosine	68 ± 31 μM	n.d.

TABLE II

Effect of nucleosides on Na$^+$-dependent uridine transport

LLC-PK$_1$ monolayers (3-day-old) were preincubated with 10 μM NBMPR for 10 min and the uptake of 5 μM [^{3}H]uridine (30 min) at 22°C measured in the presence of Na$^+$ or NMG$^+$ transport media as described in Materials and Methods. The indicated nucleosides (final concentration 100 μM) were added simultaneously with [^{3}H]uridine. Na$^+$-dependent uridine influx was defined as the difference in transport rates between Na$^+$ and NMG$^+$ transport media. The results (± S.D.) of two separate experiments are shown.

Inhibitor	Na$^+$-dependent uridine influx (pmol/mg protein per min)	
	Expt. 1	Expt. 2
None	0.67 ± 0.09	0.33 ± 0.11
Inosine	0.18 ± 0.005	0
Guanosine	0.09 ± 0.01	0.061 ± 0.009
Adenosine	0.07 ± 0.006	0.037 ± 0.005
Thymidine	0.77 ± 0.27	0.23 ± 0.013
Cytidine	0.64 ± 0.045	0.25 ± 0.031

in the presence of NMG$^+$ transport media. The kinetic parameters for equilibrium-exchange influx were approximately 6-fold higher than the zero-*trans* influx parameters (apparent K_m 680 ± 130 and 91 ± 43 μM; V_{max} 8.2 ± 1.7 and 1.4 ± 0.5 pmol/mg protein per s for equilibrium exchange and zero-*trans* influx, respectively).

Na$^+$-dependent nucleoside transport

The effect of various nucleosides on Na$^+$-dependent uridine transport was assessed in monolayers of LLC-PK$_1$ cells that had been preincubated with 10 μM NBMPR to block the Na$^+$-independent facilitated-diffusion nucleoside transporter (Fig. 2). The results of Table II show that Na$^+$-dependent uptake of uridine was inhibited by adenosine, inosine and guanosine but not by cytidine or thymidine. Long incubation times of 30 min were necessary for these experiments because of the low transport activity of the Na$^+$-dependent carrier. This raises the possibility that inhibition was occurring at the level of phosphorylation and not transport. Such as possibility can be discounted since different kinases are involved in the phosphorylation of the above group of nucleosides [26]. These results thus suggest that uridine and purine nucleosides but not other pyrimidine nucleosides are substrates for the Na$^+$-dependent nucleoside transporter of LLC-PK$_1$ cells. The data in Fig. 6 support this conclusion by demonstrating that the uptake of 5 μM guanosine was greater in the presence of NaCl media compared to NMG$^+$ media, whereas the rate of 5 μM thymidine influx was similar in both transport media. As was the case for uridine influx, no apparent Na$^+$-dependent guanosine flux was observed in the absence of preincubating cells with 10 μM NBMPR (initial rates of 11.5

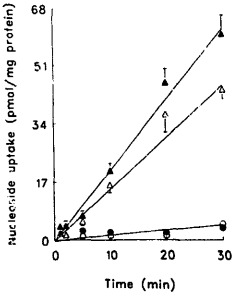

Fig. 6. Uptake of guanosine and thymidine by LLC-PK$_1$ monolayers. The uptake of 5 μM guanosine and thymidine by LLC-PK$_1$ monolayers (4-day-old) at 22°C preincubated with 10 μM NBMPR was determined at the indicated times in the presence of 140 mM NaCl (solid symbols) or 140 mM NMG^+ (open symbols). Symbols: guanosine, ● and ○; thymidine, ▲ and △.

$\pm$ 0.5 and 13.0 $\pm$ 1.6 pmol/mg protein per min in the presence of Na^+ and NMG^+, respectively).

Discussion

The data presented in this paper suggests that the routes by which nucleosides are taken up by cultured LLC-PK$_1$ renal epithelial cells appear to be mainly an NBMPR-sensitive process, a Na^+-dependent transport component specific for uridine and all purine nucleosides we have tested and a small transporter-independent route. Influx of uridine was blocked by > 95% by NBMPR. NBMPR inhibited uridine influx with a K_i value of 2.7 $\pm$ 0.75 nM and no evidence of an NBMPR-insensitive nucleoside carrier was obtained as has been observed in many other cultured cell lines [1–3,5]. Uridine influx by the NBMPR-sensitive route was saturable (apparent $K_m \sim 34$ μM at 22°C) and inhibited by both purine and pyrimidine nucleosides suggesting a broad specificity. Inhibition by NBMPR was associated with high-affinity binding of [^{3}H]NBMPR to the cells. Nucleosides blocked [^{3}H]NBMPR binding but the inhibition values were higher than the K_i values observed for inhibition of zero-*trans* uridine influx (Table I). Such discrepancies have been noted previously [24,25]. However, the K_m for equilibrium-exchange influx of formycin B (680 $\pm$ 130 μM) was similar to the inhibition constant of NBMPR binding by formycin B (455 μM). Similar agreement between the K_m values for equilibrium exchange uridine influx and the K_i values for specific NBMPR binding have been observed for mammalian erythrocytes [24,25].

The above properties of the NBMPR-sensitive nucleoside carrier in LLC-PK$_1$ cells are similar to those reported in other cultured cells with the following exceptions. First, the kinetic properties of nucleoside transport in cultured cells have either been assumed or demonstrated in a few cases to be totally symmetrical [1]. In contrast, uridine transport in human and guinea pig erythrocytes exhibits differential 'mobility' of the loaded and empty carrier (the kinetic parameters of equilibrium exchange are greater than those of zero-*trans* influx) [1,2,5,27,28]. Similarly, the present results demonstrate that the rate of formycin B transport in LLC-PK$_1$ cells is markedly stimulated when the transporter is operating in the equilibrium exchange mode compared to zero-*trans* (apparent K_m 680 $\pm$ 130 and 91 $\pm$ 43 μM; V_{max} 8.2 $\pm$ 1.7 and 1.4 $\pm$ 0.5 pmol/mg protein per s for equilibrium exchange and zero-*trans*, respectively). Within experimental error the V_{max}/K_m ratio was similar for both zero-*trans* and equilibrium exchange modes of carrier operation, a property consistent with the 'simple' transporter [29]. The estimated turnover number for the NBMPR-sensitive transporter in LLC-PK$_1$ cells of 10 uridine molecules/site per s is an order of magnitude less than that observed for the transporter in a variety of mammalian erythrocytes but similar to the estimate of the turnover number in other cultured cells [30]. It should be noted that NBMPR has the capacity to diffuse across cell membranes and thus potentially bind to intracellular binding-sites as well as those on the plasma membrane. If this was to occur with LLC-PK$_1$ cells then the above estimate of the turnover number would be too low.

The second major difference between LLC-PK$_1$ cells and other cultured cell lines appears to be in the apparent high affinity of cytidine for influx on the carrier. In Novikoff cells, the apparent K_m for influx is between 2 and 3 mM [23], which compares with a K_i estimate of 64 $\pm$ 12 μM in the present study. This difference in the two cell lines is most likely due to the different kinetic properties of the nucleoside carriers in the two cell types. The nucleoside transporter in Novikoff cells appears to exhibit equal mobility of the loaded and unloaded forms [1,31] while the mobility of empty and substrate-loaded carrier clearly differ in LLC-PK$_1$ cells (see Discussion above). Thus, the present results, together with the recent data indicating that the affinity of cytidine for the human erythrocyte nucleoside transporter also differs in the zero-*trans* and equilibrium exchange mode [32], demonstrate that cytidine exhibits a much wider range of affinity values for the equilibrative nucleoside transporter in mammalian cells than previously believed.

The second mediated route for the transport of nucleosides by LLC-PK$_1$ cells was a minor Na^+-dependent system that accounted for less than 5% of the total uridine flux at low concentrations of uridine (5

μM). Indeed, this active transporter became only apparent when LLC-PK_1 cell were incubated in the presence of NBMPR. Under such conditions efflux via the NBMPR-sensitive equilibrative transporter was blocked but permeant still entered the cells via the NBMPR-resistant concentrative transport system. In view of the low activity of the Na^+-dependent system few detailed studies on the kinetic properties of the carrier were possible. Nevertheless, limited substrate specificity studies suggest that the Na^+-dependent nucleoside transporter in LLC-PK_1 cells is identical to the N1 system described for both mouse intestinal cells and bovine renal brush-border membrane vesicles that is specific for uridine and purine nucleosides [14,33]. No evidence for the presence of the Na^+-dependent N2 nucleoside transport system in LLC-PK_1 cells was found.

The virtual absence of Na^+-dependent nucleoside transport activity in LLC-PK_1 cells was totally unexpected in view of the commonly held view that LLC-PK_1 cells are a good model for proximal tubule transport functions [15,17]. However, recent evidence suggests that LLC-PK_1 cells express characteristics typical of the late proximal tubule [16,34,35]. In particular, of the two distinct sodium-dependent D-glucose transporters arranged sequentially along the length of the proximal tubule, LLC-PK_1 cells express only the late proximal tubular D-glucose transport system [34,35]. It is therefore possible that the low expression of Na^+-dependent nucleoside transport in LLC-PK_1 cells reflects a change in the transport characteristics of the proximal tubule in vivo. Indeed, we have now demonstrated that brush-border membrane vesicles from the outer medulla of porcine kidney (late proximal tubule) exhibit a much reduced Na^+-dependent nucleoside transport activity compared to the cortical brush-border membrane vesicles (early proximal tubule) (Fig. 3).

In conclusion, the major nucleoside transport route in LLC-PK_1 renal epithelial cells derived from the late proximal tubule is a broad-specificity NBMPR-sensitive nucleoside transporter. This result, together with the low activity of Na^+-linked nucleoside transport activity in outer renal medulla brush-border vesicles and LLC-PK_1 cells, suggests that nucleoside transport across the late proximal tubule cells proceeds mainly via a facilitated-diffusion mechanism. In contrast, preliminary studies with OK cells (opossum kidney renal epithelial cells from the early proximal tubule) [36] have demonstrated marked Na^+-dependent nucleoside transport activity.

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