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Na^+-dependent and -independent uridine uptake in an established renal epithelial cell line, OK, from the opossum kidney

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The characteristics of Na^+-dependent and Na^+-independent uridine uptake at 22°C were determined for monolayers of OK renal epithelial cells. The majority of uridine influx in subconfluent to early confluent (day 1 postconfluency) OK monolayers was mediated via a facilitated-diffusion pathway (apparent K_m 160 ± 41 μM, V_{max} 610 ± 100 pmol/mg protein per min). This system was inhibited with high affinity by nitrobenzylthioinosine (NBMPR) (IC_{50} value 1.5 nM) and by purine and pyrimidine nucleosides. Specific [^{3}H]NBMPR binding sites were detected in OK monolayers (apparent K_d 0.67 ± 0.25 nM, B_{max} 90 ± 19 fmol/mg protein) yielding a turnover number for the carrier of 112 uridine molecules/site per s at 22°C. Na^+-dependent uridine uptake was minor in subconfluent OK monolayers, but increased 8-fold with time after confluency reaching a stable plateau at 8 days postconfluency. Inhibition of Na^+-dependent 1 μM uridine uptake by inosine, guanosine, adenosine and uridine was biphasic with approx. 40% of the total uptake inhibited with high affinity (IC_{50} value 2 to 14 μM). Concentrations of thymidine and cytidine up to 1 mM had no effect on Na^+-dependent uridine uptake and no Na^+-dependent thymidine influx by confluent OK monolayers was detected. Using cell monolayers grown on a permeable filter support, Na^+-dependent uridine uptake occurred preferentially from the apical surface. This high affinity component of Na^+-dependent uridine uptake is suggested to represent the Na^+-dependent purine preferring N1 nucleoside transporter. The Na^+/uridine stoichiometry for this system was consistent with 1 : 1. The remaining component of Na^+-dependent uridine uptake was inhibited by some nucleosides, such as guanosine and inosine, with low affinity (IC_{50} values of 0.6 to 5 mM). Other nucleosides showed little specific inhibition. We propose that this component of uridine uptake represents a mutated carrier that binds nucleosides but is defective in the translocation of permeant.

Introduction

Renal proximal tubular cells are polar cells possessing two distinct membrane surfaces [1]. Purified membrane vesicles prepared from the brush-border membrane (BBMV) of rat [2–4], rabbit [5] and bovine [6] renal cortex have been shown to possess concentrative Na^+-dependent nucleoside transporters. Detailed kinetic studies with BBMVs [2–7] have revealed the presence of a least two distinct Na^+-dependent nucleoside transporters, one system with a substrate preference for purine nucleosides and uridine (the N1 system) whereas the other carrier, N2, has a permeant specificity for pyrimidine nucleosides, adenosine and analogues of adenosine. Similar active nucleoside transport systems have also been found in other tissues and cells, such as intestinal enterocytes, murine and rat macrophages, murine spleenocytes and various lines of cultured mouse cells [8–12]. In contrast to the brush-border surface, recent work from this laboratory demonstrated that nucleoside transport by basolateral membrane vesicles of rabbit renal cortex proceeds via a non-concentrative, broad specificity facilitated-diffusion pathway that is inhibited by nanamolar concentrations of nitrobenzylthioinosine (NBMPR) [5]. This transport system is found in many cell types, the most widely studied of which is that present in human erythrocytes (for references see Refs. 15,16). A fourth nucleoside transporter is also a non-concentrative system that is insensitive to inhibition by low concentrations (nanamolar) of NBMPR [15–18].

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Abbreviations: NBMPR, 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranozylpurine (nitrobenzylthioinosine); Hepes, 4-(2-hydroxyethyl)-1-piperazinemethane sulphonic acid; EDTA, ethylenediaminetetraacetic acid; NMG^+, *N*-methyl-D-glucamine.

Although much information about the mechanism of transport can be obtained from vesicles, membrane vesicles provide little information regarding acute or chronic regulation of transport by hormones or substrates. Neither can they give any information concerning the transepithelial movement of nucleosides across the proximal tubule. It is therefore necessary to develop a whole cell model with which to study these questions. This in turn requires a cell line which possess both Na^+-dependent and Na^+-independent nucleoside transport systems.

In an earlier study, we found surprisingly that LLC-PK_1 cells, a porcine cell line widely used as a model for the proximal tubule, exhibited only low levels of Na^+-dependent nucleoside transport [19]. Indeed, at an extracellular concentration of 5 μM uridine, the Na^+-dependent component of influx represented just 3% of the total flux. Another proximal tubule continuous cultured cell line is OK derived from the American opossum [20]. Such cells have been shown to exhibit many different Na^+-dependent transporters, some of which are hormonally regulated [21,22]. The stoichiometry of 1 : 1 for Na^+/α-methyl D-glucoside transport by OK cells suggests that these cells originate from the early part of the proximal tubule [23]. In this study, we have characterized the uptake of uridine by OK cells as a first step towards studying the hormonal and substrate regulation of transepithelial transport in renal cells. OK cells were shown to express both Na^+-dependent and -independent uridine uptake pathways [1]. A preliminary report of some of these results has been published [24].

Materials and Methods

Cell culture

OK cells were obtained from Dr. J. Biber, University of Zurich and grown at 37°C in 5% CO_2 humidified air in Dulbecco's modified Eagles medium buffered with 25 mM Hepes and 10 mM $NaHCO_3$ (DMEM) and supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (DMEM/FCS). For transport studies, the cells were harvested by trypsinization (0.25% (w/v) trypsin/0.03% (w/v) EDTA) and seeded into 24-well culture plates at a density of $0.3 \cdot 10^6$ cells/well. Medium was changed three times a week and the day before the transport assay. The cells were examined for mycoplasma contamination and were determined to be free of mycoplasma. For studies on the localization of nucleoside transporters, the apical chamber of 4.2 cm^2 aluminium oxide tissue-culture filter inserts (Nunc, Gibco) were seeded with $3 \cdot 10^5$ cells/cm^2 and 2 ml of medium added to both the apical and basal sides of the insert.

Clones of OK cells were obtained by serial dilution of harvested cells such that on average there was one cell in every third well of a 96-well culture plate. Wells containing clones were then sequentially passed from a 24-well plate to 25 cm^2 flasks and finally to 75 cm^2 flasks. On reaching confluency, the cloned cells were frozen in DMEM/FCS containing 10% (v/v) DMSO and stored under liquid nitrogen.

Transport assay

Uptake of nucleosides by OK monolayers grown on plastic was measured as previously described for LLC-PK_1 monolayers with some changes [19]. Briefly, monolayers at the appropriate age were preincubated in DMEM for 60 min at 37°C to reduce the intracellular pools of nucleosides. The monolayers were then washed three times with 1 ml aliquots of K^+-HBSS at 22°C (145 mM KCl, 4.2 mM $KHCO_3$, 0.36 mM K_2HPO_4 $3H_2O$, 1.3 mM $CaCl_2$ $2H_2O$, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$ $6H_2O$, 10 mM Hepes pH 7.4). Uptake at 22°C was initiated by the addition of transport medium containing 10 μCi/ml [^{3}H]nucleoside, 0.4 μCi/ml inulin[^{14}C]carboxylic acid as an extracellular marker and non-radioactive nucleoside to the desired concentration in a salt solution. The salt solution consisted of the same components present in K^+-HBSS with the following alterations: in Na^+-HBSS, $KHCO_3$ was replaced with $NaHCO_3$ and KCl with 140 mM NaCl and 5 mM KCl; in Ch^+-HBSS, KCl was replaced with 140 mM choline chloride and 5 mM KCl, and in NMG^+-HBSS, KCl was replaced with 140 mM *N*-methyl-D-glutamine/HCl (NMG^+) and 5 mM KCl. In inhibition studies, test compounds and radiolabelled nucleoside were added simultaneously, except for NBMPR which was preincubated with the cells for at least 30 min. Uptake was terminated by aspiring off the transport solution one second before the indicated time and immediately washing three times with 1 ml aliquots of ice cold K^+-HBSS. Radioactivity associated with the monolayers at time zero was determined by using ice cold cells and transport medium. Monolayers were solubilized in 200 μl of 0.5 M NaOH and assayed for ^{3}H and ^{14}C content. Uptake values were corrected for ^{3}H in the extracellular space using the ^{14}C values. Cells in replicate cultures were counted after trypsin-EDTA detachment and the protein content determined by the method of Lowry [25].

In experiments with cells grown on the permeable filters, the radiolabelled solutions were added to the apical or basolateral surface. Uptake was terminated by aspirating the medium and rapidly immersing the filter insert successively into three 0.2 l volumes of

[1] In the context of this study, uptake is used to refer to cell associated radioactivity derived from incubating OK monolayers with [^{3}H]nucleoside.

ice-cold K^+-HBSS and drained. The monolayers were solubilized in 2% (w/v) SDS and the associated radioactivity determined by liquid scintillation counting. These studies were performed only with cells that had reached confluency. This was verified by visual inspection and measuring the increase in transepithelial resistance using the Millicell-ERS Resistance System (Millipore). All the monolayers used in this study exhibited a transepithelial electrical resistance of $> 40\ \Omega\ cm^2$.

Uridine metabolism

Metabolism of [^{3}H]uridine by OK monolayers was determined by thin layer chromatography as described previously [5,26].

[^{3}H]NBMPR binding to OK cell monolayers

OK monolayers at the appropriate age were washed three times with 1 ml K^+-HBSS and incubated in triplicate with graded concentrations of [^{3}H]NBMPR (0–10 nM) in K^+-HBSS (total volume of 1 ml). Incubations were carried out at 22°C in the absence and presence of 10 μM NBMPR. Specific binding was defined as the difference in [^{3}H]NBMPR bound under these two conditions. After 30 min, 50 μl samples of the incubation medium were retained for radioactive determinations and the monolayers washed and lysed as in the transport assay for the measurement of ^{3}H present.

Date analysis

Unless stated otherwise, all experiments were carried out in triplicate and errors given as standard deviations. Data was analyzed using the computer package 'Enzfitter' [27]. The dissociation constant (K_d) and density of binding sites (B_{max}) were determined by fitting data to a single-site model. The apparent affinity (K_m) and maximum velocity (V_{max}) for uptake were determined from the best fits to either the simple Michaelis Menten equation or a two-site model (Eqn. 3). Inhibition data were analyzed by the following equations:

One-site inhibition:

$$Y = (1 - ((I)/(I + K_i))) \cdot 100 \quad (1)$$

where Y = % of control value; K_i = concentration of inhibitor which gives 50% inhibition (IC_{50}); I = inhibitor concentration.

Two-site inhibition:

$$Y = (1 - (((1 - C_1) \cdot I/(I + K_1)) + ((C_1 \cdot I)/(I + K_2)))) \cdot 100 \quad (2)$$

where Y = % of control value; C_1 = capacity of site with IC_{50} of K_2; $1 - C_1$ = capacity of site with IC_{50} of K_1; I = inhibitor concentration.

Two-component Michaelis-Menten kinetics:

$$V = ((V_1 \cdot s)/(S + K_{m1})) + ((V_2 \cdot S)/(S + K_{m2})) \quad (3)$$

where V = initial rate; S = substrate concentration; V_1 = maximal uptake rate of component 1; K_{m1} = Michaelis-Menten constant of component 1; V_2 = maximal uptake rate of component 2; K_{m2} = Michaelis-Menten constant of component 2.

Materials

Cell culture reagents and plasticware were purchased from Gibco, Paisley, UK and ICN/Flow, High Wycombe, UK. [5,6-^{3}H]Uridine (46 Ci/mmol), inulin [^{14}C]carboxylic acid (10.7 mCi/mmol), [8-^{3}H]guanosine (5 Ci/mmol) and [G-^{3}H]NBMPR (23 Ci/mmol) were obtained from Amersham International (Amersham, UK), ICN Radiochemicals (Irvine, CA, USA) and Moravek Biochemicals (Brea, CA, USA). NBMPR, ouabain and gramicidin D were purchased from Sigma Chemical Co (Poole, UK). All other reagents were of analytical grade.

Results

Time course of uridine uptake

The time course of 5 μM [^{3}H]uridine uptake by 15-day-old OK monolayers is shown in Fig. 1. Marked stimulation of the initial rate of uridine uptake was observed when Na^+ replaced NMG^+ in the extracellular medium (14.2 ± 0.7 pmol/mg protein per min and 7.5 ± 0.9 pmol/mg protein per min, respectively). In the presence of 10 μM NBMPR and Na^+, 60% of the uptake of uridine was inhibited (6.2 ± 1.1 pmol/mg protein per min), and the rate of uptake was similar to that observed in the presence of NMG^+. Furthermore, the inhibition by NBMPR was potentiated by replacing Na^+ in the medium with NMG^+ reducing the rate of uridine uptake to a low basal level (0.7 ± 0.3 pmol/mg protein per min). The magnitude of the Na^+-depen-

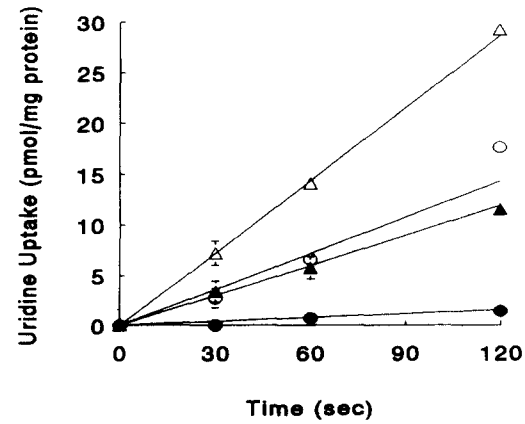

Fig. 1. Time course of uridine uptake in 15-day-old confluent OK monolayers. OK monolayers were preincubated with 10 μM NBMPR and the uptake of 5 μM uridine at 22°C determined in the presence of 140 mM Na^+ (△), 140 mM NMG^+ (○), 140 mM Na^+ plus 10 μM NBMPR (▲) and 140 mM NMG^+ plus 10 μM NBMPR (● and ▲).

dent component was not significantly different in the absence or presence of NBMPR ($P \leqq 0.05$) demonstrating that NBMPR does not affect Na^+-dependent uridine uptake in OK cells.

These results suggest the presence of two uptake routes for uridine in OK cells; an NBMPR-sensitive pathway and a Na^+-dependent system. In subsequent experiments, Na^+-independent uridine uptake was defined as the difference in uridine uptake rates in the presence and absence of 10 μM NBMPR in NMG^+ medium. The Na^+-dependent component of uridine uptake was calculated as that in the presence of Na^+ plus 10 μM NBMPR minus that in the presence of NMG^+ plus 10 μM NBMPR.

Metabolism of uridine

The fate of uridineinternalised by OK cells during an incubation period of 1 min was determined by TLC (Table I). At low [^{3}H]uridine concentrations (5 μM), 84% of the radioactivity comigrated with nucleotides decreasing to 56 and 46% at 100 and 500 μM uridine, respectively. Radioactivity associated with uracil increased with each successive increase in extracellular [^{3}H]uridine concentration whilst that associated with uridine reached a maximum of 32–34% by 100 μM extracellular uridine. Calculations based on a cell water value of 7.3 μl/mg protein [23] demonstrate that the intracellular uridine concentration at 1 min was < 10% of that in the extracellular medium at all three concentrations of uridine. As such there will be little backflux of [^{3}H]uridine during the first minute of uptake and uptake at 60 s was taken to represent the initial rate of uridine uptake.

Effect of monolayer age on uridine uptake

The activity of many transport systems in epithelial cells change as the cells develop into confluent monolayers and beyond [28]. Thus, the effect of monolayer age on the uptake of 5 μM uridine by OK cells was investigated. A small decrease in the total rate of uridine influx (Na^+-dependent plus Na^+-independent uridine uptake) from 20.0 ± 1.0 pmol/mg protein per min in sub-confluent cells to 16.4 ± 1.0 pmol/mg protein per min in 15-day-old monolayers was observed. However, Fig. 2 shows that Na^+-dependent uridine uptake increased from a low basal level of 1.0 ± 0.7 pmol/mg protein per min in sub-confluent OK cells to maximal activity (8.0 ± 1.0 pmol/mg protein per min) within 7–8 days and remained stable as the monolayer aged further. Thus, in young monolayers of OK cells uridine uptake proceeded almost exclusively via a Na^+-independent pathway, but as the monolayer aged Na^+-dependent uptake increased in activity to finally represent 40–50% of the total uridine uptake. As a result, characterization of Na^+-independent uridine uptake was performed on young monolayers (1 day post confluent), while the Na^+-dependent system was characterised using monolayers at least 7 days postconfluent.

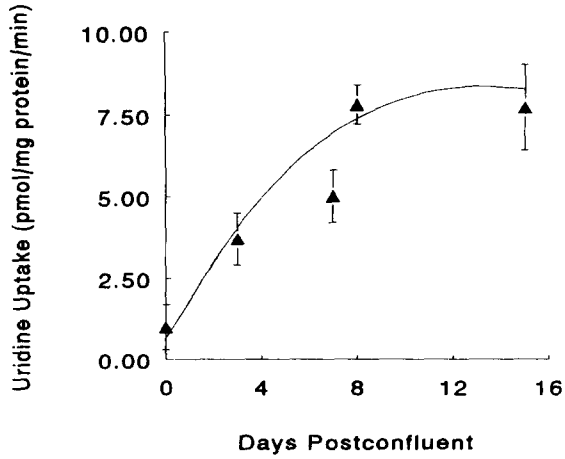

Fig. 2. Effect of monolayer age on Na^+-dependent uridine uptake in OK monolayers. OK monolayers were seeded at $3 \cdot 10^5$ cells/well and were grown for 1 day until confluency was obtained (day zero). At various times often confluency, initial rates of Na^+-dependent 5 μM uridine uptake were measured as described in the text and plotted as a function of time in culture.

Kinetic properties of Na^+-independent uridine uptake

The concentration dependence of NBMPR-sensitive, Na^+-independent [^{3}H]uridine uptake in OK cells was saturable and conformed to simple Michaelis-Menten kinetics. The mean kinetic values derived from four separate experiments were 160 ± 41 μM for the apparent K_m and 610 ± 100 pmol/mg protein per min for the V_{max}. The uptake of uridine in the presence of NBMPR was linear as a function of concentration.

NBMPR binding

Figure 3 shows that the binding of [^{3}H]NBMPR to OK monolayers was saturable. A Scatchard plot of

TABLE I

Metabolism of uridine in OK monolayers

OK monolayers (8-day-old) were incubated with the indicated concentrations of [^{3}H]uridine for 1 min and uptake of uridine terminated as described in Materials and Methods. Immediately, perchloric acid extracts of the cells were prepared and radioactivity associated with uridine, uracil and uridine nucleotides determined by TLC. More than 90% of the radioactivity loaded onto the TLC plates was recovered. The results are presented as the percentage of total counts recovered in each spot.

Extracellular uridine (μM)	Percent of ^{3}H recovered		
	uracil	uridine	nucleotides
5	2	15	83
100	11	33	56
500	20	34	46

these data (inset to Fig. 3) was linear indicating the presence of a single population of binding sites. Computer analysis of the data gave an apparent K_d of 0.80 ± 0.08 nM and a B_{max} of 120 ± 6 fmol/mg protein. The mean equilibrium binding values derived from three separate experiments were 0.67 ± 0.25 nM for the apparent K_d and 90 ± 19 fmol/mg protein for the B_{max}.

Inhibition of Na^+-independent uridine uptake

The substrate specificity of the saturable, Na^+-independent uridine uptake pathway was studied by assessing the ability of a variety of both purine and pyrimidine nucleosides and nucleoside analogues to inhibit uridine influx in OK monolayers. NBMPR completely inhibited Na^+-independent 5 μM uridine uptake with high affinity (IC_{50} of 1.5 nM). Uridine, adenosine, inosine, thymidine and formycin B also inhibited Na^+-independent uridine uptake (5 μM) with IC_{50} values of 110 ± 10, 120 ± 18, 280 ± 20, 540 ± 100 and 920 ± 30 μM respectively (mean $\pm$ S.E.). The inhibition pattern was monophasic in nature suggesting the presence of a single nucleoside transporter. Uracil had no effect on NBMPR-sensitive uridine uptake.

Na^+-dependent uridine uptake

The results of Fig. 1 showed that the rate of uridine uptake by OK monolayers in the presence of NMG^+ was significantly less than that in the presence of Na^+. Other chloride salts also resulted in a reduction in the initial rate of uridine uptake to levels not significantly different ($P < 0.05$) to the rate in the presence of NMG^+ (8.1 ± 0.4, 2.5 ± 0.3, 2.3 ± 0.05, 2.0 ± 0.2, $2.8 \pm$ 0.2 and 2.2 ± 0.3 pmol/mg protein per min for 140 mM chloride salts of Na^+, NMG^+, Li^+, K^+, Rb^+ and choline respectively at 5 μM uridine). In further experiments, the effect of a reduction in the electrochemical sodium gradient across the OK cell plasma membrane on Na^+-dependent uridine uptake was examined. Cells were preincubated for 60 min with either the Na^+/K^+ ionophore gramicidin D (10 μg/ml) [29] or the Na^+/K^+-ATPase inhibitor ouabain (1 mM) [30]. Both of these compounds caused a 50–60% reduction in Na^+-dependent 5 μM uridine influx (5.6 ± 0.3, 2.2 ± 0.6 and 2.9 ± 0.5 pmol/mg protein per min for control, gramicidin D and ouabain treated cells, respectively).

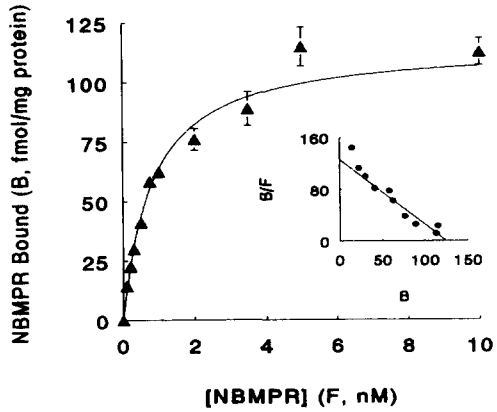

Fig. 3. Concentration dependence of specific [^{3}H]NBMPR binding to OK monolayers. OK monolayers were incubated with graded concentrations of [^{3}H]NBMPR in the presence and absence of 10 μM NBMPR for 30 min at 22°C. Specific binding of [^{3}H]NBMPR (total binding in absence of 10 μM NBMPR minus the binding in the presence of 10 μM NBMPR) is plotted as a function of the free [^{3}H]NBMPR concentration. Binding constants were determined by non-linear, least squares fit of the data and gave a K_d value of 0.80 ± 0.08 nM with a B_{max} of 120 ± 6 fmol/mg protein. The inset shows a Scatchard plot of the data.

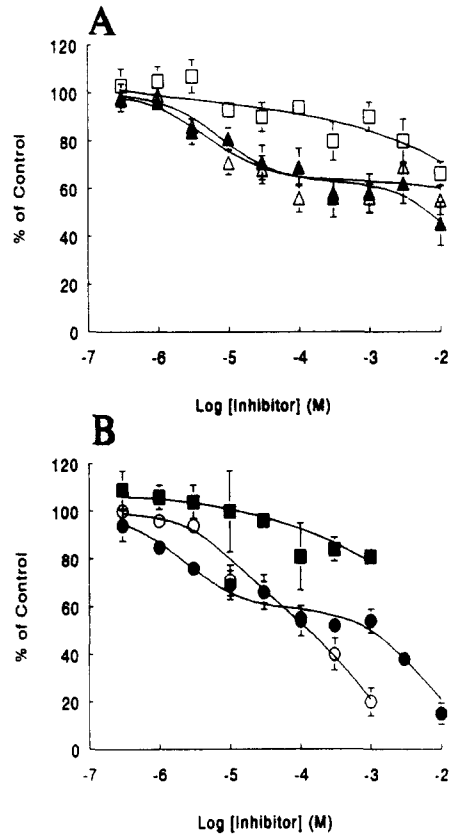

Fig. 4. Effect of nucleosides on Na^+-dependent uridine uptake in OK monolayers. OK monolayers were incubated simultaneously with 1 μM [^{3}H]uridine and varying concentrations of competing nucleoside and the initial rate of Na^+-dependent uridine uptake determined. Results are expressed as a % of control Na^+-dependent uridine uptake and represent the pooled data of a number of separate experiments. Panel A shows the dose response profile for uridine (△, $n = 14$), adenosine (▲, $n = 7$) and thymidine (□, $n = 2$). Panel B shows the dose response profile for inosine (●, $n = 6$), guanosine (○, $n = 7$) and cytidine (■, $n = 2$). All curves were fitted according to Eqn. 2. n represents the number of separate experiments.

Inhibition of Na^+-dependent uridine uptake

The ability of a variety of nucleosides to inhibit Na^+-dependent uptake of 1 μM [^{3}H]uridine in OK cell monolayers was examined (Fig. 4 and Table II). Uri-

dine, adenosine, guanosine and inosine showed biphasic dose response curves that were best described by a two site inhibition equation (see Eqn. 2 Materials and Methods). One component representing approx. 40% of the total Na^+-dependent uridine uptake at 1 μM exhibited a high-affinity with IC_{50} values in the range 2–14 μM for these nucleosides. The second component of uptake was of low sensitivity to inhibition with IC_{50} values ranging from 0.59 to 180 mM. The potency of inhibition of [^{3}H]uridine uptake by non-radioactive uridine at concentrations greater than 1 mM was similar to that observed with mannitol, a non-transported sugar, suggesting that the inhibitory effects may be due to non-specific osmotic effects (data not shown). Cytidine and thymidine failed to inhibit Na^+-dependent uridine uptake in the μM range (Fig. 4), whereas formycin B showed an apparent monophasic dose response inhibition profile with an IC_{50} value of 230 μM. Consistent with the lack of inhibition by thymidine was the finding that no Na^+-dependent uptake of 5 μM [^{3}H]thymidine was detected. The above inhibition data may suggest that OK cells possess more than one Na^+-dependent nucleoside transport system with a high specificity for purine nucleosides. To test this possibility of more than one N1 type of nucleoside transporter in OK cells, additive inhibition experiments were carried out, where inhibition by one nucleoside is analyzed in the absence and presence of a low, fixed concentration of a second nucleoside. Fig. 5 shows the results of such an experiment where the concentration dependence of guanosine inhibition was determined in the presence and absence of 50 μM inosine. In the presence of inosine, the high affinity component of guanosine inhibition was abolished, indicating that both inosine and guanosine inhibit the same transporter with high affinity. Similar experiments were performed on the effect of both guanosine and inosine on uridine and formycin B inhibition. In all cases, inosine and guanosine abolished the high-affinity component of inhibition of the second nucleoside consistent with the notion of a single, high-affinity Na^+-dependent nucleoside carrier in OK cells that accepts purine nucleosides and uridine as permeants. This substrate specificity is similar to the N1 nucleoside carriers described in other tissues and cells [2,4–7,9–14].

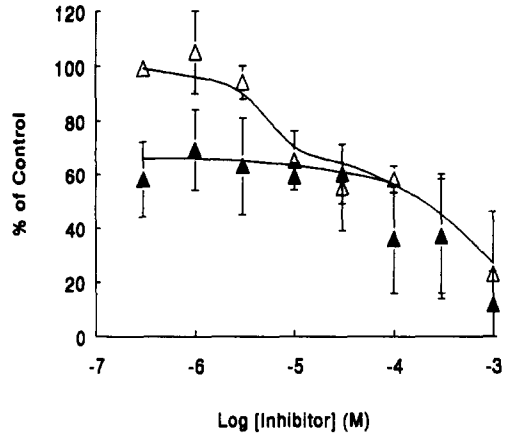

Fig. 5. Additive inhibition of Na^+-dependent uridine uptake in OK monolayers. Na^+-dependent uridine uptake (1 μM) was measured in the presence of increasing concentrations of guanosine in the presence (▲) or absence (△) of 50 μM inosine. Results are expressed as a percentage of the control flux (1.65 ± 0.35 pmol/mg protein per min) and the curves fitted according to Eqn. 1 or 2.

TABLE II

Effect of nucleosides on Na^+-dependent uridine uptake by OK monolayers

The initial rate of Na^+-dependent uridine uptake was determined in the presence of test compounds added simultaneously with [^{3}H]uridine (1 μM) to OK monolayers. The resulting inhibition curves were plotted as shown in Fig. 4 and analyzed according to Eqs. 1 and 2. The best fit data for either a single site of inhibition or for a two component inhibition curve are shown. Values represent the mean ± S.E. (*n*). NI, no inhibition.

Inhibitor	Component 1 – high affinity		Component 2 – low affinity
	IC_{50} (μM)	proportion (%)	IC_{50} (mM)
Adenosine	5.0 ± 2.0 (7)	37	> 1.0 (7)
Guanosine	14 ± 5.7 (7)	40	0.59 ± 0.27 (7)
Inosine	2.1 ± 0.5 (6)	40	5.0 ± 0.7 (6)
Formycin B	230 ± 16 (6)	100	
Uridine	7.9 ± 2.3 (14)	37	180 (2)
Cytidine	NI	0	> 1 (2)
Thymidine	NI	0	61 ± 28 (3)
Mannitol	NI	0	300 (2)

Kinetics of Na^+-dependent uridine uptake

Following these observations, the kinetics of high-affinity [^{3}H]uridine uptake were examined with respect to Na^+ and uridine concentrations. The initial rate of Na^+-dependent [^{3}H]uridine uptake (5 μM) as a function of the extracellular Na^+ concentration was determined in the absence and presence of 200 μM inosine. In both cases, uridine uptake increased in a hyperbolic manner with Hill coefficients of 1.01 ± 0.32 (4) and 0.92 ± 0.19 (3) in the absence and presence of inosine, consistent with a sodium/uridine stoichiometry of 1 : 1. In addition, the K_{Na} (concentration of Na^+ required to give half maximal Na^+-dependent nucleoside uptake activity) was also similar in the absence and presence of inosine (63 ± 5 (4) and 55 ± 6 (3) mM, respectively).

Similar experiments were performed where the Na^+ concentration was constant but the uridine concentration varied. Fig. 6 shows the concentration dependence of Na^+-dependent uridine uptake in the absence and presence of 100 μM inosine. Total uptake of uridine was not saturable in the concentration range studied but in the presence of inosine a small saturable compo-

nent of uptake was revealed. Despite the poor signal to noise ratio, the mean kinetic constants at 22°C from three separate experiments were 26 ± 6 μM for the apparent K_m with a V_{max} of 12 ± 3 pmol/mg protein per min. In other experiments, the concentration dependence of Na^+-dependent guanosine uptake was studied and analyzed using an equation describing a one-site model or a two-site model (Eqn. 3). This latter analysis revealed a high affinity site (apparent K_m 15 ± 2 μM; V_{max} 10.8 ± 3.0 pmol/mg protein per min; $n = 3$) and a low affinity site ($K_m > 500$ μM). Attempts to obtain a more accurate estimate of the low affinity K_m value by extending the guanosine concentration to 1 mM were not possible due to the signal-to-noise ratio being too low at such high concentrations. A single site model also fitted the data equally well with an apparent K_m of 52 ± 13 μM and a V_{max} of 47 ± 15 pmol/mg protein per min.

In order to determine if the two components of [^{3}H]uridine uptake revealed in the above experiments were due to the presence of more than one cell type, OK cells were cloned by limited dilution. Eight clones were obtained of which four showed biphasic uridine inhibition of [^{3}H]uridine uptake, similar to that found in the parent cell line. Hence, the two components of uptake could be present in the same cell.

Polarization of Na^+-dependent uridine uptake

The polarity of Na^+-dependent and Na^+-independent uptake in OK cells was determined using cell monolayers grown on permeable filter inserts. Table III shows that uridine uptake from the apical surface is composed of both a Na^+-dependent component and a Na^+-independent system that is blocked by NBMPR. The absolute rate of uridine uptake for cells grown on filters was approx. 2.5-fold higher than for cells grown on plastic (compare Table III and Fig. 1). However, the relative contribution of the Na^+-dependent and Na^+-independent components of uridine uptake were not dependent on the choice of matrix used to grow the cells on. In contrast to the apical surface, uridine uptake from the basolateral surface was not dependent on the presence of sodium and furthermore was totally blocked by 10 μM NBMPR (Table III).

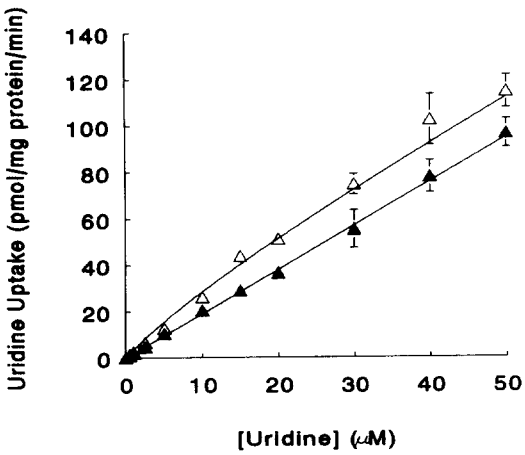

Fig. 6. Concentration dependence of Na^+-dependent uridine uptake in OK monolayers. The initial rate of Na^+-dependent uridine uptake by OK monolayers at 22°C was assessed as a function of extracellular uridine in the absence (△) and presence (▲) of 100 μM inosine. Na^+-dependent uridine uptake in the absence of inosine was analyzed according to Eqn. 3 and gave an apparent K_m of 30 ± 1 μM and a V_{max} of 15 ± 1 pmol/mg protein per min for the high-affinity system sensitive to inhibition by inosine (100 μM).

TABLE III

Sidedness of uridine transport by confluent monolayers of OK cells grown on filters

OK cells were seeded on permeable filters as described under Materials and Methods. On day 10, uridine uptake (5 μM, 60 s) was initiated by the addition of transport medium to either the apical or basolateral side of the cell. The values are the means ± S.D. of three separate determinations.

Uptake from	In the presence of	Uptake (pmol/mg protein per min)
Apical side	Na^+	34 ±4.5
	Na^+ + 10 μM NBMPR	17 ±2.1
	NMG^+	16 ±0.2
	NMG^+ + 10 μM NBMPR	0.5 ± 0.2
Basolateral	Na^+	11 ±2.1
	Na^+ + 10 μM NBMPR	−1.1 ± 0.7
	NMG^+	8.3 ± 0.2
	NMG^+ + 10 μM NBMPR	−0.7 ± 0.7

Discussion

Na^+-dependent nucleoside transport had been demonstrated in brush-border membrane vesicles from the renal cortex of a variety of species [2–7]. The present study extends this work and describes the results of a series of experiments that characterize nucleoside transport in cultured OK renal epithelial cells, a cell line that is believed to have originated from the early part of the proximal tubule [20–23]. Uptake of uridine consisted of three components; a Na^+-dependent process, an NBMPR-inhibitable system and a non-saturable process representing simple diffusion of uridine across the cell membrane. The relative contributions of the two apparent uptake systems varied as a function of culture age. Sub-confluent monolayers of OK cells transported uridine predominantly (> 95%) via the NBMPR-sensitive nucleoside transport system. However, expression of the Na^+-dependent component of uridine uptake increased as the cells reached confluency and continued to increase after cell proliferation had ceased, representing about 50% of the total uptake component at an extracellular uridine concentration of 5 μM (Fig. 2). A similar pattern of expression

of Na^+-dependent adenosine influx in IEC-6 intestinal epithelial cells has also been observed [31]. As a result of this developmental change in the relative contributions of the two systems, characterization of Na^+-independent NBMPR-sensitive uptake was performed on young monolayers (1-day-old), whereas the Na^+-dependent component was characterized with monolayers at least 7 days old.

The NBMPR-sensitive component of uridine influx in OK cells exhibited many similarities to that found in rabbit renal cortex basolateral membrane vesicles [5] and many other cells [15,16 for reviews]. Influx at 22°C was saturable (K_m approx. 160 ± 41 μM), inhibitable by a variety of purine and pyrimidine nucleosides with an order of potency generally similar to that observed in other cells and blocked by NBMPR (IC_{50} value of approx. 1.5 nM). Inhibition by NBMPR was associated with the presence of high-affinity NBMPR binding sites in OK monolayers. Assuming one NBMPR binding site represents a single functional transporter site, then using the mean values for the maximal NBMPR-sensitive uridine uptake and the maximal [^{3}H]NBMPR binding sites, the turnover number is estimated to be 112 uridine molecules/site per s at 22°C. This value is similar to that found for the NBMPR-sensitive nucleoside transporter with uridine as substrate in erythrocytes from various species [32] and a variety of other cultured cells [33], but an order of magnitude greater than that observed for LLC-PK_1 cells [19], normal rat culture kidney cells [33] and rabbit renal basolateral membrane vesicles [5]. These differences suggest that the NBMPR-sensitive facilitated-diffusion nucleoside carriers can be characterised by high or low turnover numbers.

The second route for the uptake of uridine into OK monolayers was absolutely dependent on the presence of Na^+ and represented approx. 50% of the total uridine uptake into post confluent cells. However, the properties of this component of uptake were complex and appear to consist of at least two separate systems. A number of lines of evidence are consistent with this proposal. First, the purine nucleosides, adenosine, guanosine and inosine inhibited Na^+-dependent uridine uptake in a biphasic manner with inhibition constants differing by up to 1000-fold (Fig. 4). Similar biphasic inhibition profiles were also observed for self-inhibition by uridine whereas other pyrimidine nucleosides showed little inhibition eg thymidine and cytidine (Fig. 4). Furthermore, additive inhibition experiments (Fig. 5) demonstrated that the high-affinity component of inhibition of Na^+-dependent uridine uptake by the purine nucleosides was blocked in the presence of a second purine nucleoside. These results strongly suggest that the high-affinity component of inhibition observed with the purine nucleosides and uridine represents the properties of a single transporter. Second, the kinetic properties for the concentration dependence of Na^+-dependent uridine and guanosine uptake were also consistent with two components; a high-affinity system with a K_m estimate of 26 ± 6 and 15 ± 2 μM for uridine and guanosine, respectively, and a low affinity component ($K_m > 500$ μM). Third, gramicidin D and ouabain, both compounds that dissipate the electrochemical Na^+ gradient across the plasma membrane, only partially (approx. 50%) inhibited Na^+-dependent uridine uptake. These results suggest that only part of the uridine uptake process is dependent on the sodium gradient although total uptake can be completely abolished by the removal of sodium from the transport medium. From these results we propose that the high-affinity Na^+-dependent uridine uptake process resembles the N1 active nucleoside transporter described in bovine renal brush border membrane vesicles and murine enterocytes [6,11]. But, what is the nature of the low-affinity component of uridine uptake?

The inability of uridine to inhibit its own uptake whereas other nucleosides such as inosine and guanosine gave 100% inhibition was a curious phenomenon. A number of possible explanations for this finding were tested and rejected including activation of a paracellular transport route, *trans*-stimulation of Na^+-dependent uridine uptake by intracellular uridine and transport of uridine via the Na^+-dependent glucose carrier. A further possibility is that the low-affinity uridine uptake component represents a Na^+-dependent nucleoside binding site. The partial inhibition of Na^+-dependent uridine uptake by gramicidin D and ouabain is consistent with the notion of a nucleoside binding site that requires simply the presence of Na^+ in the medium and not a Na^+ gradient across the OK cell. In addition, inhibition of this Na^+-dependent component by a number of nucleosides indicates that it represents a site mediated process requiring specific binding. This putative Na^+-dependent binding site exhibited medium affinity for some nucleosides such as guanosine and formycin B while only a low affinity for others such as thymidine, cytidine and uridine. Such a substrate specificity is similar to that of the Na^+-dependent N2 nucleoside transporter with the exception that the order of affinities for the nucleosides is reversed. Thus, it is tempting to assign the Na^+-dependent site as a N2 transporter which is defective in the translocation of permeant but not in the binding of permeant. Such a situation is not unique and has been described in OK cells with respect to the interaction of D-glucose with the Na^+-dependent hexose carrier [23].

The sidedness of the nucleoside transport systems in OK cells was studied using epithelial cells grown on filters. Our present findings indicate that Na^+-dependent uridine uptake is specifically located on the apical membrane, a finding consistent with earlier studies

demonstrating the presence of Na^+-dependent nucleoside transport activity in renal brush-border membrane vesicles [2–6]. Uridine uptake at the basolateral membrane surface was not dependent on sodium and totally blocked by NBMPR. Such a polarized arrangement of nucleoside transporters with a high-affinity transporter at the apical surface and a low-affinity carrier at the basolateral side would favour transepithelial movement of nucleosides. The polarity studies also demonstrated that Na^+-independent uridine uptake was detectable at both membrane surfaces. This finding, together with the apical localization of Na^+-dependent uridine uptake, strongly suggests that the results obtained with OK cells grown on plastic do reflect the properties of the apical membrane surface and are not due to inappropriate cell culture conditions.

In conclusion, OK cells possess an NBMPR-sensitive facilitated-diffusion nucleoside transporter and an active Na^+-dependent N1 nucleoside carrier that exhibits a high-affinity for purine nucleosides and uridine and is localized in the apical membrane. It is also possible that a mutated Na^+-dependent N2 nucleoside transporter is present in OK cells that permits the binding of permeant but not the translocation of substrate across the cell membrane.

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