Characterization of a sodium-dependent concentrative nucleobase-transport system in guinea-pig kidney cortex brush-border membrane vesicles

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The characteristics of hypoxanthine transport were examined in purified brush-border membrane vesicles isolated from guinea-pig kidney. Hypoxanthine uptake in the vesicles was specifically stimulated by both Na^+ and an inside-negative potential, resulting in a transient accumulation of intravesicular hypoxanthine. Na^+ -dependent hypoxanthine influx was saturable (apparent K_m $4.4 \pm 2.1 \mu\text{M}$, V_{max} $128 \pm 29 \text{ pmol/min per mg of protein at } 100 \text{ mM NaCl and } 22^\circ\text{C}$). Guanine, thymine, 5-fluorouracil and uracil inhibited hypoxanthine uptake (K_i values $1\text{--}30 \mu\text{M}$), but adenine and the nucleosides inosine and thymidine were without effect. Guanine competitively inhibited Na^+ -dependent hypo-

xanthine influx, suggesting that it was a substrate for the active nucleobase transporter in guinea-pig renal membrane vesicles. A sigmoidal dependence between hypoxanthine influx and Na^+ concentration was obtained (K_{Na} $13 \pm 2 \text{ mM}$; Hill coefficient, h , 2.13 ± 0.14), suggesting that at least two Na^+ ions are transported per hypoxanthine molecule. This system differs from the Na^+ -nucleobase carrier in cultured LLC-PK₁ renal cells, which has a stoichiometric coupling ratio of 1:1. These results represent the first demonstration of an active electrogenic nucleobase carrier in renal apical membrane vesicles.

INTRODUCTION

The reabsorption of a wide variety of organic solutes from the glomerular filtrate in mammalian renal proximal tubules is achieved by active Na^+ /solute co-transport across the brush-border membrane of tubular cells, and subsequent facilitated diffusion out of cells into the interstitial space [1,2]. To date, few studies on nucleobase transport by renal proximal tubules have been performed. Furthermore, the mechanisms by which the kidney handles these compounds is likely to be an important factor affecting the pharmacology, toxicology and disposition of therapeutic nucleobase analogues, such as the widely used antitumour agent 5-fluorouracil [3,4].

Recently we studied the transport of nucleobases by a cultured renal epithelial cell line, LLC-PK₁ [5]. We demonstrated the operation of a unique high-affinity Na^+ -dependent nucleobase-transport mechanism in the apical membrane of these cells, with a Na^+ :hypoxanthine coupling stoichiometry of 1:1, and a permeant specificity for the pyrimidine nucleobases uracil, 5-fluorouracil and thymine and the purine base guanine (K_i values $3\text{--}6 \mu\text{M}$). This system differed somewhat from the Na^+ -dependent pyrimidine transporter described previously in rat small intestine [6,7], which had a 22-fold higher K_m for 5-fluorouracil and only weakly interacted with guanine. In addition, LLC-PK₁ cells were also shown to possess a facilitated-diffusion purine nucleobase carrier in the basolateral membranes [5], with similar properties to the purine nucleobase carrier of human erythrocytes [8]. The different transport properties of the two membrane surfaces of LLC-PK₁ cells are consistent with a physiological role of the epithelia in nucleobase reabsorption from the tubular lumen into the blood. However, to date no studies on the nucleobase-transport properties of renal membrane vesicles have been performed to establish the physiological importance of the active nucleobase transporter observed in LLC-PK₁ cells. In the present study, we demonstrate that an

electrogenic Na^+ -dependent nucleobase-transport system is present in guinea-pig renal brush-border membrane vesicles (BBMVs), with properties similar to the carrier described in LLC-PK₁ cells, except that the Na^+ :hypoxanthine coupling stoichiometry is 2:1. A preliminary report of some of these results has been published [9].

EXPERIMENTAL

Preparation of guinea-pig cortical BBMVs

Male Dunkin–Hartley white guinea pigs (200–500 g) were killed by cervical dislocation, and the kidneys were removed, decapsulated and placed on ice. BBMVs were prepared by a modification of a Mg^{2+} precipitation procedure described previously [10]. The kidneys were sectioned transversely to reveal tissue zones, and the outer cortex was dissected and homogenized in 65 ml of a medium containing 300 mM mannitol, 5 mM EGTA and 12 mM Tris/HCl (pH 7.4) by using a Polytron on setting 5 for $4 \times 30 \text{ s}$. An equal volume of ice-cold water and MgCl_2 to a final concentration of 12 mM were then added, and the homogenate was left on ice for 15 min. All centrifugation steps were performed at 4°C . The homogenate was centrifuged at $2500 g$ for 15 min and the resultant supernatant was re-centrifuged at $30000 g$ for 30 min. The pellet was suspended in 300 mM mannitol/5 mM Tris/HCl, pH 7.4 (suspension medium), and the suspension was homogenized with a glass/Teflon Potter homogenizer (10 strokes at 1500 rev./min) and centrifuged at $30000 g$ for 30 min. Purified vesicles were resuspended in an appropriate volume of suspension medium and were used on the same day of preparation. The purity of the membrane preparation was monitored by determining the enrichment of alkaline phosphatase, a specific apical membrane enzyme marker, compared with the initial homogenate [11]; the enrichment was on average 8-fold. Protein was assayed as described by Lowry et al. [12], with BSA as a standard.

Hypoxanthine and guanine transport measurements

[³H]Hypoxanthine and [³H]guanine uptake at 22 °C by guinea-pig renal BBMVs was measured by a modification of the rapid-quench/rapid-filtration method used to determine nucleoside uptake in BBMVs [13]. The only difference was that the ice-cold stop solution did not contain phlorrhizin. Control experiments established that no loss of radioactivity from the vesicles occurred during the filtration step.

The intravesicular volume of the BBMVs was determined from the distribution ratio at equilibrium (90 min) of 3-*O*-methyl-D-[³H]glucose and was found to be similar in the presence of Na⁺ and choline⁺ (~ 1 µl/mg of protein).

Data analysis

All transport experiments were carried out in triplicate unless otherwise stated. The errors given in the Tables are S.E.M. and those in the Figures are S.D. In least-squares fits to the data points were weighted according to the inverse of their relative experimental errors. Non-linear regression analysis of data was performed by using appropriate equations and the computer program Enzfitter (Elsevier Biosoft).

Materials

[G-³H]Hypoxanthine (2.3 Ci/mmol) and 3-*O*-methyl-D-[1-³H]-glucose (2.74 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). [8-³H]Guanine (14 Ci/mmol) was purchased from Moravsek Biochemicals (Brea, CA, U.S.A.). Dilazep was generously given by Hoffman-La Roche (Basel, Switzerland). 6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosyl-purine (NBMPR), dipyridamole, papaverine and phlorrhizin were obtained from Sigma Chemical Co. (Poole, Dorset U.K.). All other reagents were analytical grade.

RESULTS AND DISCUSSION

Time course and Na⁺ specificity of hypoxanthine uptake

The time course of hypoxanthine uptake (4 µM) by guinea-pig renal BBMVs is shown in Figure 1. The uptake of hypoxanthine in the presence of an inwardly directed 100 mM NaCl gradient was markedly stimulated (~ 15-fold) compared with its uptake in the absence of Na⁺ [100 mM choline chloride gradient (out > in)]. Uptake occurred with a transient intravesicular accumulation of hypoxanthine (overshoot phenomenon), which reached its maximum (~ 2-fold above equilibrium value) at between 20 and 60 s and thereafter decreased to an equilibrium value (determined after a 90 min incubation time interval), indicating hypoxanthine efflux. In the presence of Na⁺, but in the absence of an electrochemical gradient, i.e. Na⁺ at equilibrium across the membrane, no overshoot of intravesicular hypoxanthine was observed [Figure 1 (▲)]. In a similar time-course experiment using [³H]guanine, an inwardly directed Na⁺ electrochemical gradient resulted in an overshoot of intracellular guanine, which was not observed in the presence of choline chloride (results not shown). These results indicate that guinea-pig renal BBMVs are capable of catalysing the concentrative uptake of hypoxanthine and guanine by coupling nucleobase entry to the chemical gradient of Na⁺ via a co-transport mechanism. There was no significant difference between the equilibrium values of hypoxanthine uptake in the presence of

Na⁺ and choline⁺, demonstrating that there was little or no difference in the size and integrity of the vesicles under these conditions.

When Na⁺ in the extravesicular medium was replaced by 100 mM gradients of KCl, RbCl, CsCl and LiCl (Table 1), no stimulation of hypoxanthine uptake was observed, demonstrating the high specificity of the Na⁺ requirement of hypoxanthine uptake by these vesicles. In further studies, Na⁺-dependent hypoxanthine influx was defined as the uptake in the presence of NaCl minus that in the presence of choline chloride. Na⁺-stimulated hypoxanthine uptake increased linearly with time for the first 20 s of incubation, and thus a 10 s time interval was chosen to determine initial transport rates. Extrapolation of the initial linear portion of the Na⁺-stimulated time course to zero time corresponded closely to zero uptake, indicating that there was no substantial binding (assuming that binding was rapid) to the membranes. Furthermore, after a 90 min incubation period intravesicular hypoxanthine accumulated to equilibrium levels only, demonstrating no significant binding or metabolic trapping.

Figure 1 Time course of hypoxanthine uptake by guinea-pig BBMVs

Membrane vesicles were resuspended in either 300 mM mannitol/5 mM Tris/HCl (pH 7.4) (● and ○) or 100 mM mannitol/100 mM NaCl/5 mM Tris/HCl (pH 7.4) (▲). [³H]Hypoxanthine influx (4 µM) was initiated by addition of 10 µl portions of the vesicle suspension to 20 µl of media containing (final concns.) [³H]hypoxanthine and 100 mM NaCl (●) or 100 mM choline chloride (○), or 100 mM NaCl (▲) at 22 °C.

Table 1 Effect of various univalent cations on hypoxanthine influx by guinea-pig renal BBMVs

Membrane vesicles (10 µl portions) were incubated with 20 µl of a medium containing 100 mM of the chloride salt listed in the Table, 5 mM Tris/HCl (pH 7.4) and 2 µM [³H]hypoxanthine (final concns.) at 22 °C for 10 s. Values are means ± S.D. of triplicate estimates.

Cation	Hypoxanthine influx (pmol/10 s per mg of protein)
Na ⁺	11 ± 2
Choline ⁺	2.6 ± 0.3
K ⁺	2.4 ± 0.5
Rb ⁺	2.2 ± 0.1
Cs ⁺	2.9 ± 0.8
Li ⁺	2.5 ± 0.2

Figure 2 Effect of membrane potential on hypoxanthine uptake by guinea-pig renal BBMVs

Membrane vesicles were prepared in either 300 mM mannitol/5 mM Tris/HCl (pH 7.4) (a) or 150 mM mannitol/50 mM K₂SO₄/5 mM Tris/HCl (pH 7.4) and 12 μ g of valinomycin (Val)/mg of protein (preincubated for 1 h) (b). The incubation media were either (final concns.) 2 μ M [³H]hypoxanthine and 100 mM NaSCN (●) or 50 mM Na₂SO₄ (○) (a) or 2 μ M [³H]hypoxanthine/150 mM mannitol/50 mM Na₂SO₄ (●) or 50 mM mannitol/50 mM Na₂SO₄/50 mM K₂SO₄ (○) (b).

Effect of membrane potential on hypoxanthine uptake

The coupled unidirectional transport of positively charged Na⁺ ions and uncharged hypoxanthine molecules is predicted to be electrogenic, and as a consequence sensitive to the membrane electrical potential. Thus the membrane-potential dependence of hypoxanthine uptake by guinea-pig renal BBMVs was examined by using two different but complementary approaches. Figure 2(a) compares the time course of 2 μ M [³H]hypoxanthine influx in the presence of either a 100 mM NaSCN gradient or a 50 mM Na₂SO₄ gradient. Both the initial rate of hypoxanthine influx and the magnitude of the transient overshoot were increased significantly in the presence of the more permeant SCN⁻ anion compared with that in the presence of SO₄²⁻.

The alternative approach was to pre-load vesicles with 50 mM K₂SO₄ in the presence of valinomycin (12 μ g/mg of protein) and examine the time course of uptake of 2 μ M [³H]hypoxanthine from a K⁺-free medium containing 50 mM Na₂SO₄ (Figure 2b, curve A) and a medium containing 50 mM K₂SO₄ and 50 mM Na₂SO₄ (Figure 2b, curve B). Curve A shows the time course of hypoxanthine uptake in the presence of both an inwardly directed

Figure 3 Concentration-dependence of Na⁺-dependent hypoxanthine transport by guinea-pig renal BBMVs

Membrane vesicles (a) were incubated with [³H]hypoxanthine (final concns. 0–15 μ M) at 22 °C in the presence of 100 mM NaCl (●) or choline chloride (○). Initial rates of transport were calculated from the uptake at 10 s. The Na⁺-dependent flux was taken as the rate in the presence of NaCl minus that in the presence of choline chloride for each concentration of substrate and plotted in (b).

Na⁺ concentration gradient and an inside-negative valinomycin-induced K⁺ diffusion potential (in > out). Compared with curve B [Na⁺ gradient (out > in) and K⁺ at equilibrium across the membrane], the initial rate of hypoxanthine uptake and the magnitude of the overshoot were significantly enhanced. Equilibrium uptake values were not significantly different, suggesting that the increased uptake of hypoxanthine was not attributable to a difference in the intravesicular volume. Curve B also demonstrates that a Na⁺ chemical gradient alone is sufficient to drive the concentrative uptake of hypoxanthine. These results, together with the data on the effect of anions (Figure 2a), support the view that Na⁺-dependent hypoxanthine transport is electrogenic, involving the net translocation of positive charge.

Concentration-dependence of Na⁺-dependent hypoxanthine transport

The initial rate of zero-trans hypoxanthine influx at 22 °C by guinea-pig renal BBMVs was measured as a function of the external [³H]hypoxanthine concentration (0–15 μ M) in the presence of 100 mM NaCl or 100 mM choline chloride. Figure 3(a) shows that the total uptake of hypoxanthine in the presence of Na⁺ could be resolved into a linear and a saturable component. The Michaelis-Menten kinetic constants of saturable Na⁺-dependent hypoxanthine transport (Figure 3b), determined after subtraction of the linear component (estimated in the presence of choline chloride), were $2.1 \pm 0.2 \mu\text{M}$ for the apparent K_m , with a V_{max} of $146 \pm 10 \text{ pmol/min per mg of protein}$. The mean values from three separate experiments were $4.4 \pm 2.1 \mu\text{M}$ and

Table 2 Effect of nucleobases and nucleosides on Na⁺-dependent hypoxanthine influx by guinea-pig renal BBMVs

Influx of [³H]hypoxanthine (10 s) was initiated by addition of 10 μ l portions of membrane vesicles to 20 μ l of a medium containing (final concns.) 2 μ M [³H]hypoxanthine, 100 mM NaCl or choline chloride and the test compound at 22 °C. The Na⁺-dependent component of uptake was calculated as the difference in the rate of influx in the presence and absence of Na⁺. IC₅₀ values determined by non-linear regression analysis were converted into K_i values by using the equation $K_i = IC_{50}/(1 + L/K_m)$, where values for L and K_m were taken as 2 and 4.4 μ M. Values are means $\pm$ S.E.M. of three separate experiments.

Inhibitor	Apparent K_i value (μ M)
Uracil	30 $\pm$ 4
5-Fluorouracil	20 $\pm$ 6
Thymine	7.6 $\pm$ 3
Guanine	1.8 $\pm$ 0.7
Adenine	No inhibition at 100 μ M
Inosine	No inhibition at 100 μ M
Thymidine	No inhibition at 100 μ M

Figure 4 Effect of guanine on Na⁺-dependent hypoxanthine influx by guinea-pig renal BBMVs

The reciprocals of Na⁺-dependent hypoxanthine transport rates (the difference in the rate of influx in the presence and absence of Na⁺) at hypoxanthine concentrations of 2.7 (∇), 0.93 ($\blacktriangle$), 0.48 ($\blacksquare$) and 0.23 ($\bullet$) μ M are plotted against the respective concentrations of inhibitor (guanine). Apparent K_i = 0.8 μ M.

128 $\pm$ 29 pmol/min per mg protein for K_m and V_{max} , respectively. The K_m observed in this study is 28-fold lower than that reported for Na⁺-independent hypoxanthine transport by LLC-PK₁ cells and 6-fold higher than the K_m found for Na⁺-dependent hypoxanthine influx by these cells [5].

Inhibition of Na⁺-dependent hypoxanthine transport

To explore further the structural specificity of this active nucleobase transporter in guinea-pig renal BBMVs, several nucleobases, nucleosides and transport inhibitors were tested for their ability to inhibit the influx of 2 μ M [³H]hypoxanthine (Table 2). The nucleosides inosine and thymidine had no effect on Na⁺-dependent hypoxanthine uptake at 100 μ M. Of the nucleobases tested, the purine base guanine was the most effective inhibitor,

Figure 5 Na⁺-dependent hypoxanthine flux as a function of the Na⁺ concentration

[³H]Hypoxanthine (4 μ M) uptake was measured in the presence of various extravesicular concentrations of NaCl (0–100 mM) at 22 °C. Choline replaced Na⁺ iso-osmotically to obtain the various Na⁺ concentrations studied. A least-squares fit gave a K_{Na} value of 17 $\pm$ 1 mM, with h = 2.13 $\pm$ 0.14.

followed by the pyrimidine bases thymine, 5-fluorouracil and uracil, with apparent K_i values of 1.8 $\pm$ 0.7, 7.6 $\pm$ 3, 20 $\pm$ 6 and 30 $\pm$ 4 μ M respectively. The data of Figure 4 demonstrate that guanine competitively inhibited the inward transport of hypoxanthine in guinea-pig BBMVs, with an apparent K_i value of 0.8 μ M. A Cornish-Bowden plot of these data [14] was also consistent with competitive inhibition by guanine. This interaction of guanine with the Na⁺-dependent hypoxanthine carrier, and the finding that thymine, uracil and 5-fluorouracil are effective inhibitors of hypoxanthine transport activity, strongly suggest that these nucleobases are permeants for the same system. In contrast, the purine nucleobase adenine had no effect on Na⁺-dependent hypoxanthine influx at 100 μ M. This substrate specificity is similar to the Na⁺-dependent nucleobase carrier of LLC-PK₁ cells [5], except that 5-fluorouracil and uracil were 6- and 10-fold less effective inhibitors respectively, whereas guanine was a slightly more effective inhibitor. The finding that guanine has a high affinity for both the Na⁺-dependent nucleobase transporters in guinea-pig renal BBMVs and LLC-PK₁ cells [5] appears to be a specific characteristic of renal tissues. The limited data that are available on the intestinal Na⁺-dependent pyrimidine carrier indicates that guanine is not a permeant for this system [7].

The facilitated-diffusion nucleoside-transport inhibitors NBMPR (0.1 mM), dipyrindamole and dilazep (both at 1 mM) [15] caused 57, 95 and 86% inhibition, respectively, of Na⁺-dependent hypoxanthine uptake by guinea-pig renal BBMVs. The Na⁺-dependent D-glucose-transport inhibitor phlorrhizin (3 mM) [16,17], and the facilitated-diffusion nucleobase-transport inhibitor papaverine (1 mM) [8,18], also inhibited Na⁺-dependent hypoxanthine influx (78 and 82% inhibition respectively).

Na⁺ activation of hypoxanthine transport

The Na⁺:hypoxanthine coupling stoichiometry for hypoxanthine transport by guinea-pig renal BBMVs was determined by the Na⁺-activation method over the range 0–100 mM NaCl (iso-osmolarity maintained with choline chloride) at a fixed concentration of hypoxanthine (4 μ M). Figure 5 demonstrates the sigmoidal dependence of hypoxanthine transport as a function of

the Na^+ concentration, suggesting that multiple Na^+ ions are involved in the translocation of a single hypoxanthine molecule. Non-linear regression analysis of these data by using the Hill equation [14]:

$$\text{Flux} = V_{\max} \cdot [\text{Na}^+]^h / (K_{\text{Na}}^h + [\text{Na}^+]^h)$$

where K_{Na} is the $[\text{Na}^+]$ given $0.5 V_{\max}$, and h is the Na^+ :hypoxanthine coupling stoichiometry (Hill coefficient), yielded values for K_{Na} and h of 17 ± 1 mM and 1.97 ± 0.21 respectively. The mean values from three separate experiments were 13 ± 2 mM for K_{Na} and 2.13 ± 0.14 for h . This result suggests that at least two Na^+ ions are transported per hypoxanthine molecule, and differs from the 1:1 Na^+ :hypoxanthine coupling stoichiometry previously reported for hypoxanthine transport in LLC-PK₁ cells [5]. Attempts to measure the stoichiometry by the alternative 'static head method' [19] were unsuccessful, due to the relatively low hypoxanthine transport rate and thus a poor signal-to-noise ratio.

The differences in the stoichiometry and permeant affinity that are observed in LLC-PK₁ cells and guinea-pig renal BBMVs may either be due to different species and/or cell types or reflect the presence of two distinct transporters. It is noteworthy that the kidney possesses two Na^+ -dependent glucose transporters arranged sequentially along the proximal nephron segment [20,21]. The early proximal-tubular transporter has a 1:1 Na^+ coupling stoichiometry, whereas that present at the lower end of this nephron segment exhibits a 2:1 ratio.

In conclusion, the present results have demonstrated the presence of an electrogenic Na^+ /nucleobase co-transporter in BBMVs isolated from the outer cortex of guinea-pig kidney. These results confirm that the previously described active nucleobase transport in LLC-PK₁ cells [5] is of physiological significance, and provide further support for the apical location of this transport system. As such, LLC-PK₁ cells should prove useful as

a model proximal-tubular epithelium with which to study the regulation of renal nucleobase transport.

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