ELSEVIER

Biochimica et Biophysica Acta 1416 (1999) 109–118

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Nucleobase transport in opossum kidney epithelial cells and *Xenopus laevis* oocytes: the characterisation, structure–activity relationship of uracil analogues and oocyte expression studies of sodium-dependent and -independent hypoxanthine uptake

Majid Shayeghi, Robert Akerman, Simon M. Jarvis *

Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK

Received 19 August 1998; received in revised form 27 October 1998; accepted 28 October 1998

Abstract

The characteristics of hypoxanthine transport were examined in opossum kidney (OK) epithelial cells and *Xenopus laevis* oocytes. In both cell types hypoxanthine influx was mediated by two distinct transport systems: a high-affinity Na^+-dependent system and a Na^+-independent transporter. Na^+-dependent hypoxanthine transport in OK cells was saturable (K_m 0.78 ± 0.29 μM) and was inhibited by guanine, uracil, thymine and 5-fluorouracil (K_i values 0.5–7 μM), whereas adenine had no effect. Substitutions at the 2- and 4-position had a marked effect on the ability of uracil to inhibit Na^+/hypoxanthine influx by OK cells revealing that an oxo group at both the 2- and 4-positions of uracil is required for interacting with the transporter. The properties of Na^+-dependent hypoxanthine influx in oocytes were similar to those observed in OK cells. In particular, xanthine and oxypurinol inhibited hypoxanthine influx, a characteristic not observed previously for the Na^+/nucleobase carrier in pig LLC-PK_1 renal cells. Na^+-independent hypoxanthine influx in OK cells and oocytes was of a lower affinity (K_m 90–180 μM). Adenine and guanine inhibited Na^+-independent hypoxanthine flux in OK cells, but had no effect in oocytes. Injection of LLC-PK_1 mRNA into oocytes resulted in a 1.5-fold stimulation of Na^+/hypoxanthine flux over water-injected oocytes. These results reveal further heterogeneity in Na^+/nucleobase cotransporters.

Keywords: Nucleobase transport; Kidney; Uracil analog; Expression cloning; Hypoxanthine

1. Introduction

Some animal cells, such as certain brain cells, intestinal enterocytes, bone marrow and erythrocytes are incapable of de novo purine biosynthesis and utilise nucleobases derived from extracellular sources [1]. The first step of the purine salvage pathway is the translocation of purine nucleobases across the plasma membranes of mammalian cells through specific transport proteins. These transport processes are also of considerable interest since cytotoxic nucleobases may enter cells via the carriers and inhibition of transport may enhance the activity of some nucleoside and nucleobase analogues currently in use in the chemotherapy of certain neoplastic diseases and viral infections [1,2].

Although studies of nucleobase transport have been restricted to a few cell types, there is increasing

* Corresponding author. Fax: +44 (1227) 763-912l; E-mail: s.m.jarvis@ukc.ac.uk

0005-2736/99/$ – see front matter
PII: S0005-2736(98)00213-2

evidence of multiple pathways for the uptake of these compounds. Many cells, as typified by human erythrocytes, possess a medium affinity facilitated-diffusion system for purine bases (K_m of 13–180 μM), but a low affinity for pyrimidine bases (K_m of ~4 mM) [3,4]. In other mammalian cells, hypoxanthine influx is catalysed via the nitrobenzylthioinosine (NBMPR)-insensitive nucleoside transporter (*ei*), but with a low affinity [5,6], and data from studies on S49 cells suggest that at high concentrations, nucleobases interact with the NBMPR-sensitive nucleoside transporter (*es*) [7]. Recently, Na^+-dependent nucleobase transport has been demonstrated in a range of tissues and cell lines [8–13]. These systems have a high affinity for purine and pyrimidine bases (K_m of 1–30 μM). The Na^+-dependent nucleobase transporter in the pig renal epithelial cell line, LLC-PK_1 [8], appears to be distinct from that present in rabbit choroid plexus [10]. In particular, adenine, cytosine and xanthine were potent inhibitors of Na^+-dependent hypoxanthine transport in the rabbit choroid plexus, but had no effect on Na^+-stimulated influx in LLC-PK_1 cells. In addition, the stoichiometric coupling ratio between Na^+ and hypoxanthine has been estimated to be 1:1 and 2:1 in LLC-PK_1 cells and choroid plexus, respectively [8,10]. It is not known if these differences are due to species differences, or tissue differences, or reflect the presence of multiple Na^+-nucleobase transporters in animal cells. In addition, we know nothing about the molecular properties of the specific nucleobase carriers.

Expression cloning in *Xenopus* oocytes has proved particularly successful for the identification of cDNAs encoding membrane transporters, e.g. Na^+-dependent glucose and nucleoside transporters [14–16]. An important prerequisite of such studies is to have some knowledge of the endogenous transport activity of oocytes especially if this activity is relatively large compared to that resulting from the injection of poly(A)$^+$ RNA. The goals of this study were to characterise, compare and contrast nucleobase transport in OK cells, a renal epithelial cell line derived from the early proximal tubule of opossum [17], and *Xenopus laevis* oocytes, with the properties of equilibrative and Na^+-dependent nucleobase transporters in LLC-PK_1 cells. Additional studies on exploring the structural determinants for the specificity of uracil as a permeant for the OK nucleobase co-transporter were also conducted. Following these studies we sought to identify possible selective inhibitors of the oocyte hypoxanthine transporters that would allow the blocking of the endogenous hypoxanthine transport activity prior to expression of heterologous mRNA in the oocytes. Preliminary reports of some of these findings have been published [18,19].

2. Materials and methods

2.1. Cell culture

OK cells were cultured at 37°C in 5% CO_2 humidified air in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum and 2 mM glutamine as previously described [20]. Cells were subcultured for serial passage and seeded into 24-well plates at a density of 3×10^5 cells/well for experimental use. Medium was changed every 2 days and the day before the uptake assay. Cells were free of mycoplasma as determined using Hoechst 33258 stain [21].

2.2. Hypoxanthine uptake by OK monolayers

[^{3}H]Hypoxanthine influx by confluent monolayers that completely covered the culture well surface as determined from microscopic examination was measured at 22°C as described previously using phosphate-free buffers [8]. Uptake rates are expressed as per mg protein, estimated by the method of Lowry et al. [22].

2.3. Isolation of mRNA

Large-scale isolation of mRNA from cultured cells was performed according to the manufacturer's instructions using the PolyATract System 1000 purchased from Promega. The mRNA was dissolved in RNAse-free water (1 mg/ml) and stored at −70°C until use. The integrity and purity of the samples was checked by agarose gel electrophoresis.

2.4. Oocyte preparation

Oocytes were isolated and prepared for injection of

mRNA using standard procedures [23]. In brief, mature *X. laevis* were anaesthetised by placing on ice. After isolation of ovarian fragments, follicular cells were removed by treatment with 2 mg/ml collagenase (Type I, Sigma-Aldrich) and 1 mg/ml trypsin inhibitor (Type III-O, Sigma–Aldrich) in modified Barths' solution (MBS; 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 2.5 mM sodium pyruvate, 10 mM HEPES, pH 7.5) for 1 h at 22°C. After collagenase treatment, oocytes were thoroughly washed in Ca^{2+}-free MBS and healthy stage V–VI oocytes were selected and allowed to recover overnight in MBS supplemented with 100 μg/ml gentamycin sulphate, 50 μg/ml penicillin and 5% (v/v) foetal calf serum at 16°C. These oocytes (non-injected) were used to assess endogenous nucleobase transport. In other experiments, healthy oocytes were microinjected with 50 nl of mRNA or RNAse-free water using the Drummond Nanoject. Injected oocytes were maintained in MBS at 16°C with daily changes of medium prior to use in the transport studies.

2.5. Hypoxanthine uptake by X. laevis oocytes

Non-injected and injected oocytes (10 per assay point) were washed with three 1-ml aliquots of Na^+-free transport buffer (100 mM choline chloride, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.5) followed by a 30-min preincubation in the buffer at 22°C. Uptake of [^{3}H]hypoxanthine was initiated by the addition of 0.2 ml transport buffer to oocytes in the presence and absence of 100 mM NaCl. In Na^+-free transport buffer, NaCl was replaced with choline chloride. After the appropriate time intervals, oocytes were washed with six 1.0-ml aliquots of ice-cold Na^+-free transport buffer. Each oocyte was dissolved in 0.5 ml of 10% SDS and ^{3}H was assayed by liquid-scintillation counting.

2.6. Chemicals

[G-^{3}H]Hypoxanthine (27.0 Ci/mmol) and [U-^{14}C]sucrose (626 mCi/mmol) were purchased from Amersham. Foetal bovine serum was purchased from Life Technologies. All other chemicals were of analytical grade.

2.7. Data analysis

All transport experiments with cultured cells were carried out in triplicate with at least three separate preparations unless otherwise stated. The errors given presented in the figures and tables are standard errors of the mean. In least square fits to the data, points were weighted according to the inverse of their relative experimental errors and kinetic constants of transport determined using the computer programme Enzfiter (Elsevier Biosoft).

3. Results

3.1. Hypoxanthine uptake by OK monolayers

Fig. 1 shows a time course of hypoxanthine uptake (1.4 μM) by confluent OK monolayers. Hypoxanthine influx was linear for at least 60 s and was stimulated 2.6-fold when NMG^+ was replaced with Na^+ in the extracellular medium. A time interval of 20 s was used in subsequent studies to approximate the initial rates of transport. Hypoxanthine influx rates in the presence of NMG^+ were further reduced by 80% by the addition of adenine (3 mM) and papaverine (5 mM) and were similar to hypoxanthine influx rates determined in the presence of Na^+ and 2 mM hypoxanthine (Fig. 1). These results demonstrate the presence of two distinct transporters that mediate hypoxanthine uptake in OK cells: a Na^+-dependent and a Na^+-independent system. Na^+-dependent hypoxanthine influx is defined as the difference in uptake in the presence of Na^+ and NMG^+, and Na^+-independent hypoxanthine uptake as that in the presence of NMG^+ minus that in the presence of NMG^+ plus 3 mM adenine and 5 mM papaverine. Both components were shown to exhibit saturable concentration-dependent curves that conformed to simple Michaelis–Menten kinetics. The mean kinetic constants from three separate experiments (±S.E.) were 0.78 ± 0.29 and 180 ± 59 μM for the K_m and 130 ± 18 and 2840 ± 430 pmol/mg protein per min for the V_{max} for Na^+-dependent and -independent hypoxanthine uptake by OK monolayers, respectively.

The cation specificity of the Na^+-dependent system was studied by testing the effect of a variety of

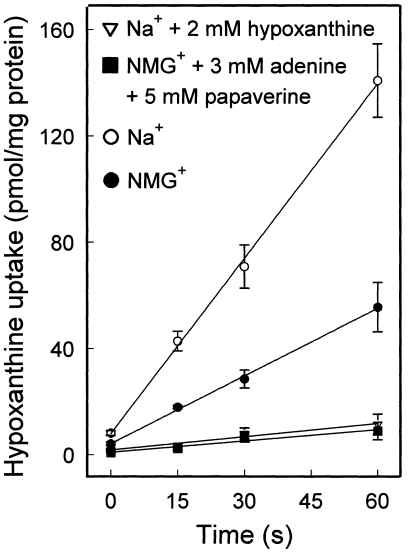

Fig. 1. Time course of hypoxanthine uptake in confluent OK monolayers. Monolayers were washed with three 1-ml aliquots of a Na^+-free medium. Hypoxanthine uptake at 22°C was initiated by the addition of transport buffer (0.2 ml) containing 1.4 μM [^{3}H]hypoxanthine and 140 mM NaCl (○), or 140 mM NMG^+ (●), or 140 mM NMG^+ plus 3 mM adenine and 5 mM papaverine (■), or 140 mM NaCl plus 2 mM hypoxanthine (▽). Uptake was terminated by washing the monolayers with three 1-ml aliquots of ice-cold Na^+-free medium. Cells were lysed with 0.5 M NaOH (0.2 ml) and counted for accumulated radioactivity. Values are the average ± S.E. of triplicate estimates.

monovalent cations (140 mM chloride salts) as a replacement of Na^+ in the transport medium. The data presented in Table 1 show that replacement of Na^+ with Li^+, NH_4^+, or K^+ resulted in at least a 2.3-fold reduction in the rate of hypoxanthine uptake and was similar to that in the presence of NMG^+. These results demonstrate the significant Na^+ specificity of hypoxanthine uptake by OK cell monolayers.

Table 1
The effect of cations on hypoxanthine uptake by OK cells

Cation	Hypoxanthine influx (pmol/mg protein per min)
Na^+	65.0 ± 1.7
Li^+	24.5 ± 0.3
K^+	27.1 ± 0.9
NH_4^+	28.7 ± 0.1
NMG^+	28.5 ± 0.2

The initial rate of 1 μM [^{3}H]hypoxanthine influx (mean ± S.E. of three independent experiments) was determined in the presence of a variety of monovalent cations (140 mM chloride salts).

The relationship between extracellular sodium concentrations (0–140 mM) and Na^+-dependent hypoxanthine uptake was examined. The Na^+ activation of the initial rate of 1 μM hypoxanthine influx revealed a hyperbolic stimulation suggestion a Na^+:hypoxanthine stoichiometry of 1:1 (Fig. 2). Non-linear regression analysis of the data using the Hill equation [24] resulted in a mean (± S.E.) K_{Na} value of 26 ± 3.9 mM and a Hill coefficient of 1.3 ± 0.3 from three separate experiments.

The potential substrate specificity of Na^+-dependent and -independent hypoxanthine transport systems in OK cells was examined by testing a wide range of compounds for their ability to inhibit the initial rate of 1 μM hypoxanthine uptake (Table 2, Fig. 3). Of the natural nucleobases tested, thymine, uracil and guanine were potent inhibitors (apparent K_i 0.6–5 μM) of Na^+-dependent hypoxanthine influx, xanthine a moderate inhibitor (apparent $K_i \sim 35$ μM) and adenine had no effect (Fig. 3 and Table 2). Additional studies where the concentrations of both thymine and [^{3}H]hypoxanthine were varied, and the data plotted as $1/v$ versus I (Dixon plot) revealed that thymine acted as a competitive inhibitor (apparent K_i value of 4.0 μM). This inhibition profile is

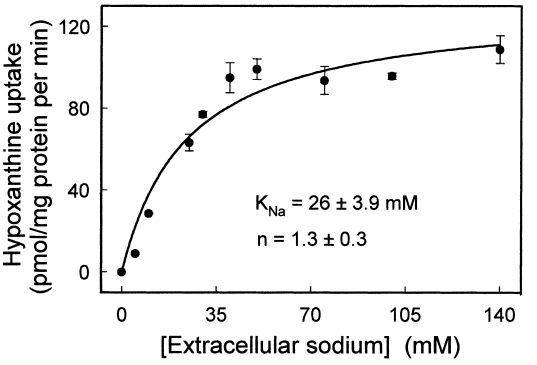

Fig. 2. Extracellular sodium dependence of hypoxanthine uptake by OK monolayers. OK monolayers were incubated for 20 s at 22°C with 1 μM [^{3}H]hypoxanthine and varying concentrations of extracellular Na^+ (0–140 mM). Osmolarity was maintained with appropriate concentrations of NMG^+. Na^+-dependent hypoxanthine flux is plotted as a function of extracellular Na^+ and the data (mean ± S.E. of triplicate values) from one representative experiment are shown. The least squares fit of the data obtained from three separate experiments (mean ± S.E.) gave a K_{Na} of 26 ± 3.9 mM with a Hill coefficient of 1.3 ± 0.3.

Table 2
The effect of a range of compounds and transport inhibitors on hypoxanthine uptake by OK cells

Test compound	Na^+-dependent apparent K_i (μM)	Ratio	Na^+-independent apparent K_i (μM)
Uracil	3.9 ± 0.2	1.0	NI
1-Substitution			
1-Methyluracil	NI	–	NI
2-Substitutions			
2-Aminouracil (isocytosine)	NI	–	NI
4-Hydroxypyrimidine (2-deoxyuracil)	NI	–	NI
2-Thiouracil	NI	–	NI
3-Substitutions			
3-Methyluracil	NI	–	NI
3-Deazuracil	> 1000	< 0.004	NI
4-Substitutions			
4-Aminouracil (cytosine)	NI	–	NI
2-Hydroxypyrimidine (4-deoxyuracil)	NI	–	NI
5-Substitutions			
5-Chlorouracil	3.9 ± 0.4	1.0	NI
5-Fluorouracil	6.2 ± 0.3	0.63	NI
5-Methyluracil (thymine)	4.2 ± 0.3	0.93	> 1000
5-Nitrouracil	> 100 μM	< 0.04	NI
6-Substitutions			
6-Methyluracil	24 ± 2.6	0.16	NI
6-Azauracil	> 1000	< 0.004	
Purine compounds			
Guanine	0.6 ± 0.04	6.5	45 ± 2.8
Xanthine	35 ± 4.9	0.11	NI
Adenine	> 100	< 0.04	85 ± 22
Oxypurinol	5.2 ± 0.9	0.75	NI
Allopurinol	NI	–	NI
Other compounds			
Uric acid	NI	–	NI
Azidothymidine	NI	–	NI
Phloridzin	Ni	–	NI
Dipyridamole	0.9 ± 0.08	4.3	> 1000
Dilazep	137 ± 42	0.028	> 1000
Nitrobenzylthioinosine	> 100	< 0.04	NI

The initial rate of 1 μM [^{3}H]hypoxanthine influx was determined in the presence and absence of Na^+ and varying inhibitor concentrations. The rates of uptake were plotted as a percentage of the control flux versus log inhibitor concentration (see Fig. 3) and IC_{50} values determined. K_i values were calculated from the equation $K_i = IC_{50}/(1+L/K_m)$, where $L = 1$ μM hypoxanthine and the K_m values for Na^+-dependent and -independent hypoxanthine influx were 0.78 and 181 μM, respectively. K_i values are the means ± S.E.M. of at least three separate experiments. NI, no inhibition (less than 10% of control values) at 100 μM. Ratio = apparent K_i for uracil/apparent K_i for compound.

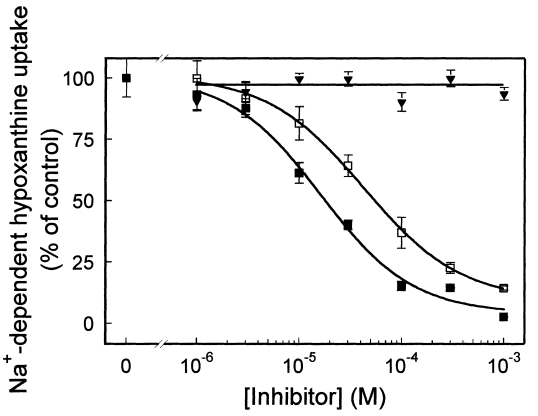

Fig. 3. Effect of adenine, 6-methyluracil and 5-chlorouracil on Na^+-dependent hypoxanthine uptake in OK monolayers. The Na^+-dependent component of 1 μM [^{3}H]hypoxanthine influx was determined in the presence of adenine (▼), 6-methyluracil (□) and 5-chlorouracil (■). The results are from a single experiment performed in triplicate (mean ± S.E.). See Table 2 for mean K_i values.

different to that observed previously with LLC-PK_1 cells where xanthine failed to inhibit Na^+-dependent hypoxanthine influx [8]. Similarly, oxypurinol was now demonstrated to inhibit Na^+-dependent hypoxanthine influx by OK cells (apparent K_i 5.2 ± 0.9 μM), but had no effect on hypoxanthine flux in LLC-PK_1 cells (Table 3). Interestingly, allopurinol, the parent compound from which oxypurinol is metabolised, had no effect on either Na^+-dependent or -independent hypoxanthine uptake. For the Na^+-independent component of hypoxanthine influx, of all the compounds tested, only guanine and adenine exhibited significant inhibition of the flux catalysed by OK cells.

To attempt to provide information on the structure–activity relationship for the interaction of nucleobases with the Na^+-dependent nucleobase carrier in OK cells, the effect of uracil analogues as inhibitors of Na^+/hypoxanthine uptake were examined. The apparent K_i values, and the ratio between the apparent K_i value for the reference compound, uracil, and the apparent K_i value for each compound screened is shown in Table 2. This ratio indicates the fold increase (values >1) or decrease (values <1) in the potency of the compound, relative to uracil. The data clearly demonstrate that substitutions of the 2- or 4-position oxo group with an amino group (isocytosine, cytosine) or elimination of one of the oxo groups (2-deoxyuracil, 4-deoxyuracil) severely decreased or abolished binding. Similarly, substitution of the oxo group at the 2-position with thiol failed to restore binding. Substitution of the hydrogen at either the 1- or 3-position of uracil with a hydrophobic group, such as methyl, also eliminated binding. Replacement of the C6 carbon of uracil with a nitrogen (6-azauracil) also decreased binding that may be related to the compound being 100% ionised under the conditions employed [25]. Substitution of the C6 hydrogen with a methyl group decreased interaction with the transporter ~6-fold. Replacement of the 5-position hydrogen with groups that are more electronegative than hydrogen (nitro > fluoro > chloro > hydrogen) generally inhibited binding to the Na^+/hypoxanthine transporter in OK cells.

3.2. Hypoxanthine uptake by X. laevis oocytes

Preliminary time course experiments of the uptake of 1 μM hypoxanthine by *X. laevis* oocytes demonstrated that uptake was linear for up to 60 min and was stimulated ~5-fold when choline was replaced with Na^+ in the transport buffer. Thus, a time interval of 20 min was chosen in subsequent experiments to determine initial rates of transport.

The selectivity of the Na^+ requirement for hypoxanthine (1 μM) influx by oocytes was studied by replacing Na^+ in the incubation medium with a variety of cations (chloride salts at 100 mM). Results in

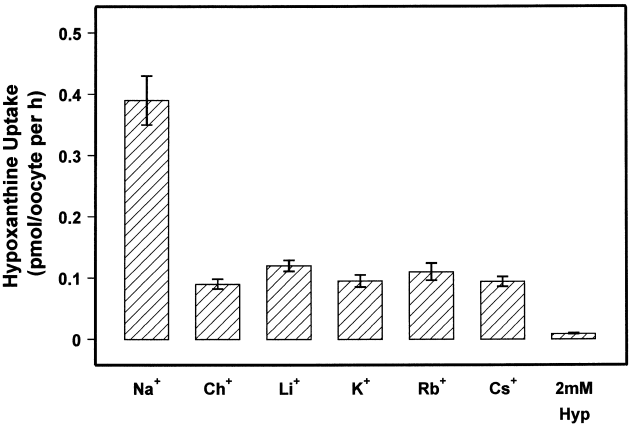

Fig. 4. Effect of cations on hypoxanthine uptake by *Xenopus laevis* oocytes. The initial rate of 1 μM [^{3}H]hypoxanthine influx (mean ± S.E. ($n = 10$)) was determined in the presence of a variety of monovalent cations (100 mM chloride salts). Uptake was also determined in the presence of sodium buffer containing 2 mM hypoxanthine (Hyp).

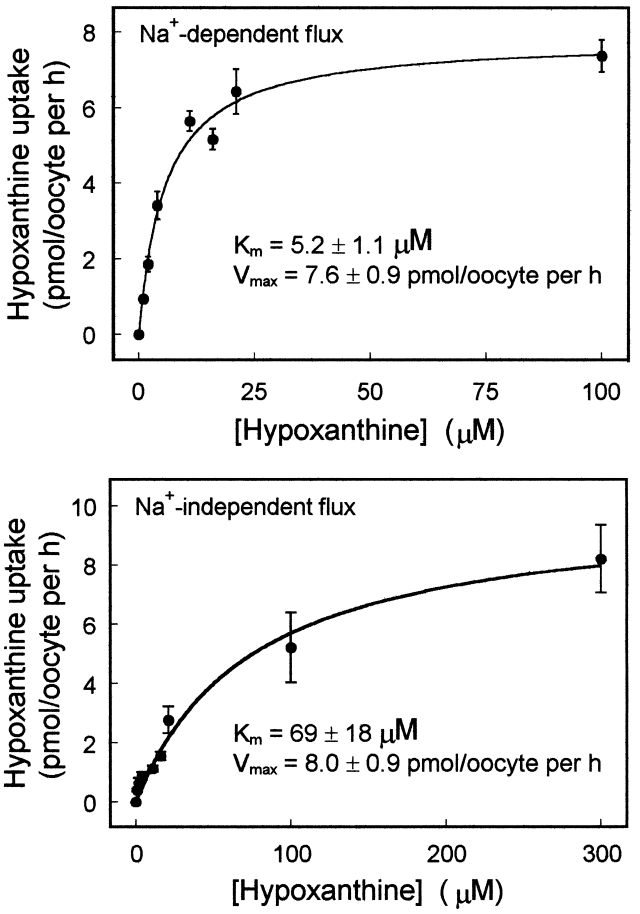

Fig. 5. Concentration dependence of hypoxanthine uptake in *Xenopus laevis* oocytes. Oocytes were incubated with varying concentrations of [^{3}H]hypoxanthine in transport buffers containing either Na^+, or choline, or choline plus 2 mM hypoxanthine. The Na^+-dependent and -independent flux components of hypoxanthine uptake were determined and plotted with the kinetic constants shown on the figures. The mean (±S.E.) K_m and V_{max} values for Na^+-dependent and -independent hypoxanthine flux from three separate experiments were 5.8±1.0 and 87±23 μM and 7.5±0.5 and 7.3±0.9 pmol/oocyte per h, respectively.

Fig. 4 show that when Na^+ was replaced by choline, Li^+, K^+, Rb^+ or Cs^+, hypoxanthine uptake was reduced by up to 4-fold. These results demonstrate the high Na^+ specificity of hypoxanthine uptake by *Xenopus* oocytes, and in further studies Na^+-dependent hypoxanthine transport was defined as the uptake in the presence of Na^+ minus that in the presence of choline. Fig. 4 also revealed the presence of a second mediated hypoxanthine transport system in *Xenopus* oocytes by the observation that addition of 2 mM hypoxanthine to the Na^+ transport buffer reduced 1 μM [^{3}H]hypoxanthine influx to levels below that observed in Na^+-free buffer.

Fig. 5 shows the concentration dependence of Na^+-dependent and -independent hypoxanthine influx by *Xenopus* oocytes. Hypoxanthine influx by both components was saturable and conformed to simple Michaelis–Menten kinetics for a single saturable process. The mean (±S.E.) kinetic constants from three separate experiments were 5.8±1.0 and 87±23 μM for the apparent K_m with V_{max} values of 7.5±0.5 and 7.3±0.9 pmol/oocyte per h for Na^+-dependent and -independent hypoxanthine influx.

The inhibition profile of the Na^+-dependent hypoxanthine transport component in OK cells showed distinct differences from that observed for LLC-PK_1 cells. Thus, we tested the effects of a variety of nucleobases on both Na^+-dependent and -independent hypoxanthine influx by *Xenopus* oocytes. Table 3 shows that xanthine and oxypurinol, nucleobases that inhibited Na^+-dependent hypoxanthine influx in OK cells, but not LLC-PK_1 cells, also inhibited Na^+/hypoxanthine cotransport in oocytes. The kinetics of uracil inhibition of Na^+-dependent hypoxanthine uptake were further analysed. Fig. 6 shows the results of an experiment where the effect of varying the concentration of uracil on Na^+-dependent hypoxanthine flux by oocytes at differing concentrations of the permanent is plotted as $1/v$

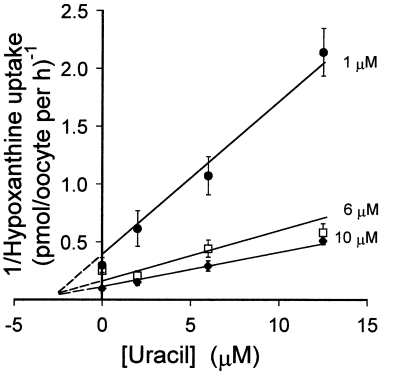

Fig. 6. Dixon plot of uracil inhibition of Na^+-dependent hypoxanthine uptake by *Xenopus laevis* oocytes. The reciprocals of the rate of Na^+-dependent hypoxanthine uptake (mean±S.E. of triplicate values) (1 μM (●), 6 μM (□) and 10 μM (♦)) are plotted against graded concentrations of uracil. Apparent K_i value ~2.5 μM.

versus I (Dixon plot). The plot was consistent with competitive inhibition and gave an apparent K_i value of 2.5 μM. None of the compounds listed in Table 3 inhibited Na^+-independent hypoxanthine influx by oocytes at 100 μM.

3.3. Expression of heterologous Na^+/hypoxanthine transporters in oocytes

The different inhibition profiles of Na^+-dependent hypoxanthine influx by *Xenopus* oocytes and LLC-PK_1 cells raised the possibility that the endogenous Na^+-dependent component of hypoxanthine uptake could be selectively blocked by addition of xanthine. Table 4 shows the results of this approach and the subsequent expression of Na^+-dependent hypoxanthine uptake following injection of LLC-PK_1 poly-$(A)^+$ RNA. Total Na^+-dependent hypoxanthine uptake was stimulated 1.5-fold in oocytes microinjected with LLC-PK_1 mRNA compared to water-injected oocytes. Addition of 100 μM xanthine reduced Na^+-dependent hypoxanthine transport in both control water-injected oocytes and mRNA-injected oocytes, but a significant difference between the two samples was still apparent. Unfortunately, subsequent experiments with different batches of both oocytes and mRNA revealed that the expression of Na^+-dependent hypoxanthine transport was variable.

Table 3
Apparent K_i values for inhibition of Na^+-dependent hypoxanthine uptake in *Xenopus laevis* oocytes and LLC-PK_1 renal epithelial cells

Test compound	Apparent K_i (μM)	
	Oocytes	LLC-PK_1
Thymine	5.5 ± 0.7	3.5 ± 0.2[a]
Xanthine	54.0 ± 6.1	NI[a]
Uracil	6.6 ± 1.1	2.9 ± 0.2[a]
5-Fluorouracil	5.9 ± 1.0	3.3 ± 0.4[a]
Oxypurinol	35.0 ± 4.2	NI
Adenine	NI	NI
2-Thiouracil	NI	ND
6-Azauracil	NI	ND
Uric acid	NI	NI

NI, no inhibition at 100 μM; ND, not determined. The initial rate of Na^+-dependent hypoxanthine uptake (1 μM) was plotted as a function of inhibitor concentration and IC_{50} values determined as described in Table 2. K_i values are the means ± S.E.M. of three separate experiments.
[a]Data taken from [8].

Table 4
Expression of the Na^+-dependent nucleobase transporter of LLC-PK_1 cells in *Xenopus laevis* oocytes

Assay conditions	Na^+-dependent hypoxanthine uptake (pmol/oocyte per h)	
	mRNA-injected	Water-injected
Control	3.32 ± 0.14	2.15 ± 0.3
+100 μM Xanthine	0.82 ± 0.03	0.54 ± 0.05

Oocytes were injected with 50 nl of poly$(A)^+$ RNA (1 μg/μl) from LLC-PK_1 cells or water and incubated for 3 days at 16°C. Uptake of 1 μM [^{3}H]hypoxanthine was determined at 22°C for 20 min in the presence and absence of unlabelled xanthine in the presence of 100 mM NaCl or 100 mM choline chloride. Na^+-dependent hypoxanthine uptake rates (flux in the presence of NaCl minus that in the presence of choline chloride) are presented (mean ± S.E.M. for 10 oocytes).

4. Discussion

The present investigation extends a previous study [8] demonstrating the presence of Na^+-dependent and -independent hypoxanthine transport in renal derived cultured cells. In addition, the structure–activity relationship of the permeant site of the Na^+/hypoxanthine transporter has been explored and preliminary attempts to functionally express a renal nucleobase transporter in *X. laevis* oocytes are presented.

The cultured renal epithelial cell line established from early proximal tubules of the opossum (designated OK) kidney [17] was shown to possess Na^+-dependent hypoxanthine transport activity. The transporter had a similar high-affinity (K_m of 0.78 ± 0.29 μM) to that determined for the Na^+-dependent nucleobase transporter in pig LLC-PK_1 late proximal tubule cells (K_m 0.79 ± 0.43 μM) [8]. The Na^+:hypoxanthine coupling stoichiometry in both cell types was estimated to be 1:1, but the V_{max} was approximately 9-fold higher for the Na^+/hypoxanthine cotransporter in OK cells (V_{max} of 132 ± 18 versus 15 ± 4 pmol/mg protein per min). The higher V_{max} may be of physiological significance in ensuring efficient reabsorption of hypoxanthine and other nucleobases in the early part of the proximal tubule. The differences in V_{max} values could also be due to

species differences. Hypoxanthine influx via the Na^+/hypoxanthine cotransporter in OK cells was strongly inhibited by purine and pyrimidine nucleobases including guanine, thymine, uracil and 5-fluorouracil (K_i values <10 μM), and as is the case for Na^+-dependent hypoxanthine transport in LLC-PK_1 cells adenine had no inhibitory effect on hypoxanthine uptake. Another similarity between the two cell types was the discrimination dipyridamole and dilazep showed in their inhibition of Na^+-dependent and -independent hypoxanthine flux. Both compounds were relative potent inhibitors of Na^+-dependent hypoxanthine flux. However, oxypurinol and xanthine, nucleobases that had no effect on Na^+-dependent hypoxanthine influx in LLC-PK_1 cells [8] (Table 3) did inhibit Na^+-dependent hypoxanthine uptake by OK cells (K_i values <60 μM). This result indicates further heterogeneity in Na^+-dependent nucleobase transporters to that previously reported occurring both between species and tissues [8–13,26]. Characterisation of hypoxanthine influx in *Xenopus* oocytes also demonstrated that these cells possess a Na^+-dependent hypoxanthine transport activity with properties similar to those in OK cells.

The structure–activity relationship of the permeant site of the OK cell Na^+-dependent transporter was examined by investigating the effects of a range of uracil analogues on hypoxanthine influx (Table 3). The data clearly demonstrated that an oxo group at both the 2- and 4-positions of uracil was required for the interaction of uracil with the cotransporter. Substitution of the N1 and N3 hydrogen with a hydrophobic group, e.g. methyl, also eliminated interaction of the uracil analogue with the carrier suggesting that the region of the transporter adjacent to the N1 and N3 position is either hydrophilic in nature or sterically hindered.

OK cells were also shown to take up hypoxanthine via a Na^+-independent transport system with over a 200-fold lower affinity than the Na^+-dependent transporter (K_m 180 ± 59 vs 0.78 ± 0.29 μM). As is the case for other Na^+-independent hypoxanthine mammalian transporters [1,3,4], the carrier in OK cells was preferentially inhibited by purine nucleobases. The failure of NBMPR to inhibit Na^+-independent hypoxanthine influx in OK cells suggests that the NBMPR-sensitive nucleoside transporter in these cells [20] does not mediate the uptake of hypoxanthine. Comparable studies with *X. laevis* oocytes also demonstrated that oocytes possess a mediated Na^+-independent route of hypoxanthine entry, but in contrast to the system in OK cells was not inhibited by adenine. This result suggests that hypoxanthine enters oocytes either via a novel system or via a carrier that is not a specific nucleobase transporter.

There is currently no knowledge of the molecular properties of mammalian nucleobase transporters. The *Xenopus* oocyte expression cloning strategy has been successfully applied to the cloning of membrane transport proteins especially where the level of transport activity in the oocyte is low, e.g. nucleoside uptake [15,16]. The finding that *X. laevis* oocytes possess a hypoxanthine flux rate that is approximately 20-fold the rate of nucleoside influx [15,16,27], and moreover the properties of Na^+-dependent hypoxanthine flux are similar to those in OK cells, implied that it may be difficult to detect enhancement of hypoxanthine influx in oocytes following injection of OK poly(A)$^+$ RNA. However, the differences in transport properties of Na^+-dependent hypoxanthine transport between LLC-PK_1 cells and oocytes suggested that pretreatment of oocytes with 100 μM xanthine should reduce the endogenous flux without an effect on the flux catalysed by the LLC-PK_1 hypoxanthine cotransporter. The results of Table 4 confirm that this was possible and a 1.5-fold stimulation in Na^+-dependent hypoxanthine flux was detectable. Unfortunately, in further experiments, the expression of hypoxanthine flux was variable and current experiments are attempting to reduce this variability by enriching the mRNA by size fractionation before injecting into oocytes.

In conclusion, hypoxanthine transport in OK renal epithelial cells derived from the early proximal tubule and *X. laevis* oocytes was shown to occur by both a Na^+-dependent nucleobase transport system that was different from those previously characterised and by a Na^+-independent nucleobase carrier. Functional expression of renal Na^+-dependent nucleobase transport activity was detected in oocytes following injection of mRNA, but expression was variable. Provided reproducible and higher levels of hypoxanthine flux can be achieved following microinjection, it should be possible to use the oocyte expression cloning strategy to isolate and clone Na^+-dependent nucleobase transporters.

Acknowledgements

This work was supported in part by the Medical Research Council. M.S. was in receipt of an MRC Postgraduate Studentship.

References

[1] D.A. Griffith, S.M. Jarvis, Biochim. Biophys. Acta 1286 (1996) 153–181.
[2] K. Isono, Pharmacol. Ther. 52 (1991) 269–285.
[3] B.A. Domin, W.B. Mahoney, T.P. Zimmerman, J. Biol. Chem. 263 (1988) 9276–9284.
[4] M. Kraupp, R. Marz, G. Prager, W. Kommer, M. Razavi, M. Baghestanian, P. Chiba, Biochim. Biophys. Acta 1070 (1991) 157–162.
[5] N. Ossess, J.D. Pearson, D.L. Yudilevich, S.M. Jarvis, Biochem. J. 317 (1996) 843–848.
[6] R. Marz, R.M. Wohlhueter, P.G.W. Plagemann, J. Biol. Chem. 254 (1979) 2329–2338.
[7] B. Aronow, B. Ullman, J. Biol. Chem. 261 (1986) 2014–2019.
[8] D.A. Griffith, S.M. Jarvis, J. Biol. Chem. 268 (1993) 20085–20090.
[9] P.S. Haddock, Cardiovas. Res. 30 (1995) 130–137.
[10] C.B. Washington, K.M. Giacomini, J. Biol. Chem. 270 (1995) 22816–22819.
[11] C.B. Washington, K.M. Giacomini, Phamac. Res. 13 (1996) 1276–1278.
[12] D.A. Griffith, S.M. Jarvis, Biochem. J. 303 (1994) 901–905.
[13] J.R. Bronk, J.G. Hastewell, J. Physiol (Lond.) 382 (1987) 475–488.
[14] M.A. Hediger, M.A. Coady, T.S. Ikeda, E.M. Wright, Nature 330 (1987) 379–381.
[15] Q.-Q. Huang, S.Y.M. Yao, M.W.L. Ritzel, A.R.P. Paterson, C.E. Cass, J.D. Young, J. Biol. Chem. 269 (1994) 17757–17760.
[16] M. Che, D.F. Oritz, I.M. Arias, J. Biol. Chem. 270 (1995) 13596–13599.
[17] H. Koyama, C. Goodpasture, H.M. Miller, R.L. Teplitz, A.D. Riggs, In Vitro 14 (1978) 239–246.
[18] M. Shayeghi, S.M. Jarvis, J. Physiol. (Lond.) 489P (1995) 126.
[19] R. Akerman, S.M. Jarvis, Biochem. Soc. Trans. 23 (1995) 29S.
[20] A.J. Doherty, S.M. Jarvis, Biochim. Biophys. Acta 1147 (1993) 214–222.
[21] M. Battaglia, D. Pozzi, S. Grimaldi, T. Parasassi, Biotech. Histochem. 69 (1994) 152–156.
[22] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[23] S.M. Jarvis, H.M. Thomas, in: D. Rickwood (Eds.), Methods in Molecular Biology, 2nd edn., Chapman and Hall, London, 1998.
[24] I.H. Segel, Enzyme Kinetics, John Wiley and Sons, New York, 1975.
[25] J.G. Niedzwicki, M.H. Iltzsch, M.H. el Kouni, S. Cha, Biochem. Pharmacol. 33 (1984) 2383–2395.
[26] L.S. Schanker, J.J. Jeffrey, D.J. Tocco, Biochem. Pharmacol. 12 (1963) 1047–1053.
[27] S.M. Jarvis, D.A. Griffith, Biochem. J. 278 (1991) 605–607.