Nucleoside uptake in rat liver parenchymal cells

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Rat liver parenchymal cells express Na⁺-dependent and Na⁺-independent nucleoside transport activity. The Na⁺-dependent component shows kinetic properties and substrate specificity similar to those reported for plasma membrane vesicles [Ruiz-Montasell, Casado, Felipe and Pastor-Anglada (1992) *J. Membr. Biol.* **128**, 227–233]. This transport activity shows apparent K_m values for uridine in the range 8–13 μ M and a V_{max} of 246 pmol of uridine per 3 min per 10⁶ cells. Most nucleosides, including the analogue formycin B, *cis*-inhibit Na⁺-dependent uridine transport, although thymidine and cytidine are poor inhibitors. Inosine and adenosine inhibit Na⁺-dependent uridine uptake in a dose-dependent manner, reaching total inhibition. Guanosine also inhibits Na⁺-dependent uridine uptake, although there is some residual transport activity (35% of the control values) that is resistant to high concentrations of guanosine but may be inhibited by low concentrations of adenosine. The transport activity that

is inhibited by high concentrations of thymidine is similar to the guanosine-resistant fraction. These observations are consistent with the presence of at least two Na⁺-dependent transport systems. Na⁺-dependent uridine uptake is sensitive to *N*-ethylmaleimide treatment, but Na⁺-independent transport is not. Nitrobenzylthioinosine (NBTI) stimulates Na⁺-dependent uridine uptake. The NBTI effect involves a change in V_{max} , it is rapid, dose-dependent, does not need preincubation and can be abolished by depleting the Na⁺ transmembrane electrochemical gradient. Na⁺-independent uridine transport seems to be insensitive to NBTI. Under the same experimental conditions, NBTI effectively blocks most of the Na⁺-independent uridine uptake in hepatoma cells. Thus the stimulatory effect of NBTI on the concentrative nucleoside transporter of liver parenchymal cells cannot be explained by inhibition of nucleoside efflux.

INTRODUCTION

Nucleoside uptake in mammalian cells is mediated by various transport systems, which involve at least two separate mechanisms: a Na⁺-independent facilitative transport activity and a Na⁺-dependent concentrative system [1,2]. Most cells express equilibrative nucleoside transport activities that show broad specificity and have been classically subdivided according to their sensitivity to inhibition by nitrobenzylthioinosine (NBTI). Because correlation between the presence of high-affinity NBTI-binding sites and NBTI-sensitive transport activity has often been reported in the literature, it has been suggested that this analogue binds specifically to the nucleoside carrier molecules [1]. Some specialized cell types express a concentrative nucleoside transport activity that is Na⁺-dependent and NBTI-insensitive [1,2]. This activity has been described for renal and intestinal epithelial cells and for other established cell lines derived from both epithelia [3–10]. Kinetic characterization of this transport activity provided evidence that it might correspond to several transport systems: N1 (cif), N2 (cit) and N3 (cib) [11]. N1 preferentially accepts purine nucleosides and uridine, N2 accepts pyrimidine nucleosides and adenosine, and N3 shows broad specificity. The three activities have been successfully expressed in *Xenopus laevis* oocytes [12,13]. Pajor and Wright [14], taking advantage of the sequence similarity with the SGLT1 cDNA, cloned a mammalian Na⁺-nucleoside co-transporter, SNST1, from a rabbit kidney cDNA library. Huang et al. [15] subsequently reported the cloning and functional expression of a cDNA, cNT1, encoding a high-affinity N2-type Na⁺-dependent

nucleoside transporter. Recently a new cDNA clone, SPNT, encoding a Na⁺-dependent nucleoside transport system, has been isolated from rat liver [16]. Similarly to cNT1, SPNT shows high expression in jejunum [16]. SPNT encodes a purine-preferring transporter, which might correspond to an N1-type transport activity. cNT1 and SPNT might belong to the same gene family but they do not show any sequence similarity with the SNST1 clone.

In summary, there is extensive kinetic information and some preliminary molecular characterization of those transport systems involved in the uptake of nucleosides by most mammalian cells. Nevertheless the liver, a key organ in nucleoside metabolism, has been little studied until recently. Although this organ shows a high endogenous capacity for nucleoside biosynthesis, it is extremely efficient in extracting afferent nucleosides from blood [17–20], generating intracellular concentrations far beyond the equilibrium [21]. This rules out the possibility of the involvement of a single Na⁺-independent facilitated transport system in nucleoside transport into liver parenchymal cells. Nevertheless NBTI-binding had been reported in earlier studies in liver plasma membrane vesicles [22,23]. Indeed, this analogue was extensively used as a nucleoside transport inhibitor in metabolic studies designed to determine the role of intracellular nucleosides in the control of key pathways such as glycogen synthesis and ureogenesis [24–26]. The first evidence of concentrative uptake of nucleosides was provided by using isolated rat hepatocytes and monitoring thymidine uptake [27]. Na⁺-dependent thymidine transport was found to be insensitive to inhibition by most nucleosides. It was assumed that a thymidine transport system

Abbreviations used: FCS, fetal calf serum; NBTI, nitrobenzylthioinosine; NEM, *N*-ethylmaleimide.

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with very narrow substrate specificity is expressed in liver parenchymal cells. Subsequent observations, using the isolated perfused liver technique, provided evidence of concentrative nucleoside uptake in liver cells [19]. Later, and almost simultaneously, three laboratories described for the first time the occurrence of a Na^+ -dependent agency in plasma membrane vesicles from rat liver [28–30]. This transport activity shows broad substrate specificity and a likely nucleoside-to- Na^+ stoichiometry of 1:1; it is electrogenic and is apparently functionally linked to canalicular ectonucleotidases.

To demonstrate that this transport system is expressed in liver parenchymal cells and to determine which other agencies are involved in the uptake of nucleosides, we have explored the characteristics of Na^+ -dependent and Na^+ -independent uridine uptake in isolated hepatocytes, both in suspension and in primary culture. The concentrative transport activity found in liver parenchymal cells is consistent with the existence of two agencies that may show similar affinities for uridine, but may be purine- and pyrimidine-preferring. We have also studied the effect of NBTI on the Na^+ -dependent transport and compared it with hepatoma cells. Our results strongly suggest that the use of NBTI as an inhibitor of nucleoside uptake in liver parenchymal cells is inappropriate and therefore studies performed on this basis should now be re-evaluated.

EXPERIMENTAL

Materials

[5,6- ^3H]Uridine (38.5 Ci/mmol) was purchased from Du Pont–New England Nuclear. D-[1- ^{14}C]Mannitol (57 Ci/mmol) was obtained from Amersham. NBTI and other nucleosides were obtained from Sigma and Aldrich. Collagenase A from *Clostridium histolyticum* was purchased from Boehringer Mannheim. 3-Dibutyl phthalate and bis-(3,5,5-trimethylhexyl) phthalate were from Fluka. *N*-Ethylmaleimide (NEM), monensin and ouabain were purchased from Sigma. All media used for cell culture were obtained from Whittaker. All other reagents were of analytical grade.

Isolation and primary culture of hepatocytes

Hepatocytes were isolated from fed male Wistar rats (200–240 g), by an adaptation of previous procedures, as described by Moreno et al. [31]. The liver was perfused with calcium- and magnesium-free Hanks balanced salt solution containing 0.5 mM EGTA and buffered to pH 7.3 with 50 mM Hepes. After washing off the blood, this buffer was replaced by a collagenase (9.1 m-units/ml)-containing digestion buffer (137 mM NaCl, 1.3 mM Na_2HPO_4 , 5.4 mM KCl, 0.44 mM KH_2PO_4 , 0.98 mM MgCl_2 , 0.81 mM MgSO_4 , 5 mM CaCl_2 , 5.5 mM glucose and 20 mM Hepes, pH 7.4). The isolated cells were washed several times and suspended in the medium indicated above, except for the CaCl_2 concentration, which was decreased to 1.33 mM. Mean viability was monitored by exclusion of 0.4% Trypan Blue and always exceeded 93%. When cells were used for transport measurements, they were pelleted and resuspended in a medium containing 1.3 mM K_2HPO_4 , 5.4 mM KCl, 0.44 mM KH_2PO_4 , 0.98 mM MgCl_2 , 0.81 mM MgSO_4 and either 137 mM NaCl or 137 mM choline chloride. When used for primary culture, cells were seeded on Petri tissue culture dishes (35 mm) at a mean density of 10^5 cells/cm 2 . They were cultured for 4 h in 2% fetal calf serum (FCS)-supplemented 199 medium, already containing sodium bicarbonate, glutamine and Earle's balanced salt solution. Dexamethasone (200 nM) was added during this short period to allow efficient attachment of cells to the plates. This

medium was then replaced by a similar 199 with 0.2% BSA instead of serum. An appropriate mixture of antibiotics was also used to avoid contamination.

Some preliminary studies were performed with the human hepatoma cell line HepG2. HepG2 cells were routinely cultured in a mixture of 1:1 high-glucose Eagle's minimum essential medium (EMEM) and Ham's F12 medium. EMEM contained 1% (w/v) glutamine, 1% (w/v) sodium bicarbonate, 1% (w/v) non-essential amino acid mixture, 2% (w/v) FCS and a mixture of antibiotics. Ham's F12 contained antibiotics, 1% (w/v) glutamine and 10% (w/v) NCS $^-$.

Transport measurements

Uridine uptake by isolated liver parenchymal cells in suspension was measured as described elsewhere [32]. Briefly, cells were washed and resuspended, as indicated above, in either a NaCl or a choline chloride medium. Then they were incubated in the presence of the same buffer containing [5,6- ^3H]uridine, at the indicated concentrations and with specific activities ranging from 100 to 2500 d.p.m./pmol. The mean cell concentration used during the incubation was $(0.5\text{--}1.5) \times 10^6$ cells/ml. When the incubation time had elapsed, samples (0.25×10^6 cells) were collected and added to ice-cold Eppendorf tubes, containing an upper buffer phase, an intermediate oil layer [dibutyl phthalate/bis-(3,5,5-trimethylhexyl) phthalate (3:1, v/v)] and a lower layer of HClO_4 /glycerol (1:9, v/v). The tube was immediately centrifuged (14000 g for 60 s), the supernatants were aspirated and the radioactivity of the acid extract (pellet) was measured. D-[1- ^{14}C]Mannitol was included in the incubation medium to reveal the amount of extracellular medium trapped in the acid layer. The validity of this method has recently been reviewed [33]. Uptake rates at the indicated times are expressed as pmol of uridine per 10^6 cells. Transport measurements on primary cultures of rat hepatocytes and on the human hepatoma cell line HepG2 were performed as follows. Briefly, the uptake buffer consisted of 5.4 mM KCl, 1.8 mM CaCl_2 , 1.2 mM MgSO_4 , 10 mM Hepes, 1 μM [5,6- ^3H]uridine, pH 7.4, and either 137 mM NaCl or 137 mM choline chloride. Incubation was stopped by washing three times with 1 ml of a cold stop solution consisting of 137 mM NaCl and 10 mM Hepes, pH 7.4. After quick drying of the plates, 0.5 ml of a 0.5% Triton X-100 solution was added. This extract was used for both radioactivity measurement and protein determination [34]. In this case it was not necessary to correct for an extracellular marker because the incubation medium of the monolayers was completely washed off. Uptake rates are expressed as pmol/mg of protein.

Analysis of the data

Experimental data were analysed with either the Enzfitter (Elsevier, Biosoft) or the Fig P (Biosoft, Fig.P Corporation) programs. Linear and nonlinear regression analyses were used to determine Hill coefficients and Michaelis–Menten constants respectively. Both programs gave similar kinetic values. Fitting of the data, when indicated, was achieved with the Fig P software. The goodness-of-fit index was always within 95% expected limits for a correct model for the data.

Uridine metabolism

To check whether significant metabolism of uridine occurred in our experimental conditions, we analysed the emergence of nucleoside-related metabolites as previously described [29]. Briefly, isolated liver parenchymal cells were incubated in the

presence of the radiolabelled substrate for 4 min, and aliquots were taken at different times (first sampling at 15 s). Cell extracts (100 μ l) were analysed by paper chromatography with Whatman 3MM Chr paper strips 35 cm long and 2 cm wide. The eluent was n-butanol/ammonia/acetone/water (110:7.5:30:52.5, by vol.). After chromatography for about 16 h the strips were cut at 2 cm intervals and counted for radioactivity. Commercial [5,6- 3 H]uridine was treated in the same way as the samples and used as a standard. Standards and most samples gave two peaks: a major peak, corresponding to uridine, and a small peak, which was contaminating uracil. The amount recovered was in accordance with the analysis from Du Pont. Thus no significant metabolism of uridine seemed to occur. Only isolated hepatocytes incubated for 4 min yielded two further peaks, probably corresponding to phosphorylated nucleoside derivatives and a product of uracil catabolism (result not shown). A similar analysis was performed on primary cultures of rat hepatocytes and HepG2 cells. In this case, longer periods were necessary to detect products of uridine metabolism.

Isolation of liver plasma membrane vesicles

We used plasma membrane vesicles from rat liver to monitor uridine uptake and compare nucleoside transport in this system with the results obtained with either isolated liver parenchymal cells or cell culture. A few results on plasma membrane vesicles are presented for comparative purposes only. Because the method and conditions used have been explained in detail previously [29,35,36], we refer to earlier studies for more information about the experimental procedure.

RESULTS

Nucleoside uptake by liver parenchymal cells

The time course of 0.5 μ M uridine uptake and the substrate concentration dependence of uridine transport into rat liver parenchymal cells are shown in Figure 1. Na^+ -dependent uptake was linear for at least 4 min (Figure 1a). Na^+ -independent uridine

Figure 1 Time course and substrate concentration dependence of uridine uptake into isolated hepatocytes

(a) Isolated hepatocytes were incubated in the presence of 0.5 μ M uridine for 4 min either in a NaCl (●) or a choline chloride (○) medium, as described in the Experimental section. Incubation was stopped at the indicated times and Na^+ -dependent uptake (■) was calculated by subtracting those rates measured in choline medium from those measured in a Na^+ medium. Results correspond to a representative experiment and are the means $\pm$ S.E.M. for triplicate estimations. Independent hepatocyte preparations resulted in similar time course profiles but showed certain individual variability. (b) The dependence of Na^+ -linked uridine uptake on substrate concentration was studied over the range of uridine concentrations from 0.1 to 25 μ M. The results are the means $\pm$ S.E.M. for triplicate estimations made on five independent hepatocyte preparations.

Figure 2 Effect of nucleosides and NBTI on uridine transport into liver parenchymal cells

Freshly isolated rat liver parenchymal cells were incubated, as indicated in the Experimental section, in the presence of 0.5 μ M uridine for 1 min, either in the absence or in the presence of selected nucleosides and NBTI at a concentration of 100 μ M. These compounds were added at the same time as the substrate. When indicated, uridine was added at a concentration of 100 μ M to monitor the effects of NBTI at higher substrate concentrations. Measurements were performed either in a NaCl or a choline chloride medium (Na^+ and choline media respectively). Na^+ -dependent transport was calculated by subtracting the uptake rates measured in choline medium from those determined in Na^+ medium. Results are expressed as percentages of control values (uptake rates measured in the absence of inhibitor) and are the means for triplicate estimations made on four to six independent preparations. (a) The inhibition of Na^+ -dependent uptake: basal uptake rates were in the range 1–7 pmol of uridine per min per 10⁶ cells. (b) The inhibition of uridine uptake measured in a choline chloride medium: basal uptake rates were in the range 0.7–3.5 pmol of uridine per min per 10⁶ cells.

transport was linear for at least 2 min (Figure 1a). Na^+ -dependent uridine uptake was dependent on the substrate concentration, following classical Michaelis–Menten kinetics (Figure 1b). Analysis of the experimental data gave the following K_m and V_{max} values respectively: 13 ± 2 μ M and 246 ± 17 pmol of uridine per 3 min per 10⁶ cells. *Cis*-inhibition of uridine transport into isolated hepatocytes by other nucleosides is shown in Figure 2. All the nucleosides tested, including the analogue formycin B, inhibited Na^+ -dependent uridine uptake (Figure 2a). Inosine and adenosine showed the highest inhibition of Na^+ -dependent nucleoside uptake. Cytidine and thymidine were poor inhibitors of the concentrative transport activity. Na^+ -independent uridine transport was partly inhibited by all nucleosides tested except

Figure 3 Dose-response inhibition of Na^+ -dependent uridine uptake by selected nucleosides

Isolated hepatocytes were incubated in the presence of $0.5 \mu\text{M}$ uridine for 1 min, either in a NaCl or a choline chloride medium, in the presence of increasing concentrations of selected nucleosides. Symbols are: $\square$, adenosine; $\bullet$, guanosine; $\blacksquare$, $200 \mu\text{M}$ thymidine; $\circ$, $200 \mu\text{M}$ guanosine plus $25 \mu\text{M}$ adenosine. Na^+ -dependent transport rates were calculated from the uptake measurements performed in both media (Na^+ and choline). Na^+ -dependent uptake rates in the absence of the inhibitors were in the range 2–6 pmol of uridine per min per 10^6 cells. Results are expressed as the fraction of Na^+ -dependent transport remaining after inhibition by nucleosides, and are the means $\pm$ S.E.M. for triplicate estimations made on four independent hepatocyte preparations.

Figure 4 Dose-response inhibition of Na^+ -dependent uridine uptake by inosine

Isolated hepatocytes were incubated in the presence of $0.5 \mu\text{M}$ uridine for 1 min, either in a NaCl or a choline chloride medium, in the presence of increasing concentrations of inosine (up to $200 \mu\text{M}$). Inosine concentrations are plotted on logarithmically. Na^+ -dependent transport rates were calculated from the uptake measurements performed in both media (Na^+ and choline). Na^+ -dependent uptake in the absence of the inhibitor was in the range 3–7 pmol of uridine per min per 10^6 cells. Results are expressed as the fraction of Na^+ -dependent transport remaining after inhibition by nucleosides, and are the means $\pm$ S.E.M. for triplicate estimations made on four independent hepatocyte preparations.

guanosine and cytidine (Figure 2b). When dose-response inhibition curves were performed (Figures 3 and 4), with selected nucleosides as inhibitors and measuring Na^+ -dependent uridine uptake, the following observations were made: first, adenosine exerted almost total inhibition in a dose-dependent manner (Figure 3); secondly, guanosine also inhibited in a dose-dependent manner but only partly and the residual activity could be further inhibited by low concentrations of adenosine (Figure 3). In quantitative terms, the guanosine-resistant fraction of Na^+ -

Figure 5 Effect of NEM treatment on uridine uptake in isolated hepatocytes

Isolated hepatocytes were preincubated for 30 min with or without increasing concentrations (up to $100 \mu\text{M}$) of NEM. Then $0.5 \mu\text{M}$ uridine uptake was measured either in a NaCl ($\circ$) or in a choline chloride ($\bullet$) medium. Results are the means $\pm$ S.E.M. for triplicate estimations made on two independent hepatocyte preparations.

dependent uridine transport was similar to the fraction inhibited by high concentrations of thymidine (Figure 3). Figure 4 shows the inhibition pattern of Na^+ -dependent uridine uptake by another nucleoside, inosine, expressed on a semilogarithmic basis. Inhibition by inosine was clearly biphasic, showing a 50% inhibition of Na^+ -dependent uridine transport at an inhibitor concentration of $5 \mu\text{M}$. These inhibition studies are consistent with the possibility of liver parenchymal cells expressing more than a single transport system for Na^+ -dependent nucleoside transport.

NBTI, used at a supramaximal concentration of $100 \mu\text{M}$, exerted a minor effect (17%) on the Na^+ -independent transport activity and markedly enhanced the total uptake by selectively enhancing Na^+ -dependent transport of uridine (Figure 2). This effect was still evident when uridine was used at a substrate concentration ($100 \mu\text{M}$) in the range of the reported K_m values of the equilibrative Na^+ -independent transport systems (Figure 2). This effect was analysed later in more detail.

When hepatocytes were cultured for 24 h, most cell preparations lost the Na^+ -dependent transport system. Some batches of cells retained this transport activity at markedly lower levels than those found in freshly isolated cells. Na^+ -dependent transport was preserved in suspensions of isolated hepatocytes incubated for 3 h at 37°C , but decreased in culture 4 h after plating (results not shown). Na^+ -dependent transport recovered progressively but never reached the original specific activities (results not shown).

Effect of NEM treatment on Na^+ -dependent and Na^+ -independent uridine uptake

Na^+ -dependent uridine uptake into isolated rat liver parenchymal cells was almost totally inhibited by NEM pretreatment of the cells. This effect was dose-dependent (Figure 5). Interestingly, uridine uptake in the absence of Na^+ was insensitive to NEM.

Stimulatory effect of NBTI on Na^+ -dependent uridine transport

To analyse the stimulatory effect of NBTI on the Na^+ -dependent uptake of uridine, we first determined whether this was still evident at concentrations of the analogue known to exert specific inhibition of the NBTI-sensitive nucleoside transport (in the

Figure 6 Effect of NBTI on uridine uptake in isolated hepatocytes

Uptake of 0.5 μ M uridine was monitored either with or without 10 nM NBTI treatment. NBTI was either preincubated (a) or added at the same time as the substrate (b), and uridine transport was measured either in a NaCl (hatched columns) or choline chloride (open columns) medium. Na⁺-dependent uptake (filled columns) was calculated by subtracting those rates measured in choline medium from those determined in Na⁺ medium. The results are the means $\pm$ S.E.M. for triplicate estimations made on two independent hepatocyte preparations.

Figure 7 Effect of NBTI concentration on Na⁺-dependent uridine uptake in isolated hepatocytes

Isolated hepatocytes were incubated with NBTI and 0.5 μ M uridine simultaneously. Na⁺-dependent uptake was calculated as indicated in the legend to Figure 3. NBTI was added in a range of concentrations from 1 pM to 100 μ M. Results are expressed as percentages of stimulation triggered by NBTI above control values (absence of NBTI) and are the means $\pm$ S.E.M. for triplicate estimations made on three independent hepatocyte preparations.

nanomolar range). Results shown in Figure 6 show that 10 nM NBTI equally stimulated Na⁺-dependent uridine uptake without modifying the Na⁺-independent uptake rates. Furthermore no preincubation was necessary, because NBTI, when added at the same time as the substrate, resulted in a similar stimulation, although slightly lower than in those cells preincubated for 30 min with the analogue. The effect was rapid: even 15 s after the beginning of the incubation time, the uptake rates of the Na⁺-dependent system were already enhanced (up to 2.5-fold above control values) in the presence of 10 nM NBTI, without preincubation. The magnitude of this effect was the same at all the incubation times tested (results not shown). The stimulatory effect of NBTI on the Na⁺-dependent uptake of uridine by isolated hepatocytes was dose-dependent (Figure 7). The half-maximal effect was found between 1 and 5 nM and the maximal stimulation was 140% above the control values. These assays were performed without preincubation of the cells with NBTI. To disclose the nature of the NBTI effect on the Na⁺-dependent uptake of uridine, we studied how NBTI could modify the dependence of this transport system on both uridine and Na⁺. Results are shown in Figure 8. In both experiments NBTI was

Figure 8 Effect of NBTI on the substrate concentration dependence of Na⁺-dependent uridine uptake in isolated hepatocytes

(a) Isolated hepatocytes were incubated either with (●) or without (○) 10 nM NBTI, in the presence of increasing concentrations of uridine at a fixed NaCl concentration (137 mM). (b) Isolated hepatocytes were incubated at a fixed uridine concentration (0.5 μ M) and in the presence of increasing concentrations of NaCl, up to 137 mM, with (●) or without (○) 10 nM NBTI. Results in both panels are the means $\pm$ S.E.M. for triplicate estimations made on two independent hepatocyte preparations.

added at the same time as substrate (no preincubation), which led to a marked change in substrate flux without affecting the affinity for either co-substrate, Na⁺ or uridine. $V_{\max}$ values derived from Figure 8(a) were 118 ± 20 and 286 ± 28 pmol of uridine per min per 10⁶ cells for non-treated and NBTI-treated cells respectively. K_m values were 9 ± 2 and 8 ± 2 μ M for NBTI-treated and non-treated cells respectively. These estimations were made in the presence of physiological concentrations of Na⁺ (137 mM). Maximal velocity values derived from Figure 8(b) were 8.2 ± 2.4 and 27.5 ± 5.8 pmol of uridine per min per 10⁶ cells for non-treated and NBTI-treated cells respectively. The K_{Na} was not significantly modified by NBTI (107 ± 38 mM compared with 109 ± 30 mM). For both co-substrates, maximal substrate flux was enhanced 140% above the values obtained in the absence of NBTI. Hill plots of the data from Figure 8(b) showed that the Hill coefficient was not changed due to NBTI action (1.11 compared with 0.96).

The effect of the dissipation of the Na⁺ transmembrane electrochemical gradient on NBTI action was assessed with the use of two drugs, ouabain (a specific inhibitor of Na⁺,K⁺-

Figure 9 Effect of ouabain and monensin treatment on NBTI-stimulated Na⁺-dependent uridine uptake in isolated hepatocytes

Isolated hepatocytes were treated for 30 min with 1 mM ouabain and preincubated for the last 10 min with increasing concentrations (25 and 100 µM) of monensin. Controls were preincubated for 30 min in the absence of both drugs. Then the uptake of 0.5 µM uridine was monitored either in the absence or in the presence of 10 nM NBTI. Results correspond to the Na⁺-dependent fraction of transport and are the means ± S.E.M. for triplicate estimations made on two independent hepatocyte preparations.

Table 1 Effect of NBTI on uridine transport into rat liver plasma membrane vesicles and the human hepatoma cell line HepG2

Rat liver plasma membrane vesicles (LPMV) were preincubated for 30 min either in the presence or in the absence of 100 µM NBTI. Then the uptake of 1 µM uridine was measured in a potassium thiocyanate medium, as described in the Experimental section. Results (expressed as pmol of uridine per 3 s per mg of protein) are the means ± S.E.M. for triplicate estimations made on pooled fractions from four independent vesicle preparations. Subconfluent monolayer cultures of HepG2 cells were preincubated under identical conditions either with or without NBTI for 30 min, at the indicated concentrations. Then the uptake of 1 µM uridine was determined in a choline chloride medium, as described in the Experimental section. Results (expressed as pmol of uridine per 3 min per 10⁶ cells) are the means ± S.E.M. for triplicate observations made on three independent batches of cells.

Preparation	Addition	Uridine uptake rate
LPMV	None	0.086 ± 0.015
	100 µM NBTI	0.090 ± 0.015
HepG2	None	24 ± 3
	100 µM NBTI	4 ± 0.5
	100 nM NBTI	5 ± 1.5

ATPase) and monensin (a Na⁺/H⁺ ionophore) (Figure 9). After preincubation of the cells with 1 mM ouabain, significant inhibition of both basal and NBTI-stimulated Na⁺-dependent uridine uptake occurred, although NBTI was still able to enhance uridine transport to a similar extent. Addition of increasing concentrations of monensin (up to 100 µM) did not further modify the Na⁺-dependent uptake of uridine but completely blocked the effect induced by NBTI.

The effect of NBTI pretreatment on uridine uptake rates in rat liver plasma membrane vesicles and in HepG2 cells is shown in Table 1. In accordance with the results from isolated hepatocytes, 100 µM NBTI did not modify uridine transport into liver plasma membrane vesicles when measured in a potassium thiocyanate medium. However, the human hepatoma cell line HepG2, which showed a negligible Na⁺-dependent component involved in nucleoside uptake, expressed a Na⁺-independent transport system that was highly sensitive to NBTI inhibition. No preincubation was necessary to observe this effect (results not shown). The rat

hepatoma cell line FAO also expressed NBTI-sensitive nucleoside uptake when measured under the same conditions (results not shown).

DISCUSSION

Liver parenchymal cells show a Na⁺-dependent uridine transport activity similar to that described in liver plasma membrane vesicles. The *K_m* for uridine in vesicles is in the range 6–9 µM [28,35]. Other workers, using adenosine as substrate, have reported *K_m* values of 8 µM [27] and 14 µM [29]. In the present study values of 8–13 µM for uridine are reported. *Cis*-inhibition of Na⁺-dependent nucleoside uptake by liver plasma membrane vesicles showed that inosine, guanosine, adenosine and uridine were strong inhibitors, whereas thymidine and cytidine inhibited nucleoside transport only at high concentrations [19,28–30]. In general terms a similar inhibition pattern was found in the present study when using isolated hepatocytes, although a more detailed inhibition analysis revealed that liver parenchymal cells might express more than a single Na⁺-dependent transport system. Considering the recent cloning of a purine-preferring transport system from liver cells (SPNT) [16], it is highly probable that hepatocytes express a second putative isoform of nucleoside carriers involved in pyrimidine uptake. Preliminary observations from our laboratory support this hypothesis (A. Felipe, N. Rocamora, B. del Santo, F. J. Casado and M. Pastor-Anglada, unpublished work). The expression, in a single cell type, of two different genes encoding two nucleoside transport systems with different substrate specificities is consistent with the inhibition studies discussed above.

Significant inhibition of uridine transport by other nucleosides in the choline chloride medium suggests that a Na⁺-independent agency is active in isolated rat liver parenchymal cells. The different sensitivities of the Na⁺-dependent and Na⁺-independent components of NEM inhibition support the view that these transport systems are not structurally related. Inhibition of nucleoside transport by thiol-modifying reagents such as mercurials has been reported in several other cell types [1,37,38]. The Na⁺-independent transport system has not been characterized in detail but, according to our results, might be responsible for a significant fraction of total nucleoside transport into hepatocytes and is highly resistant to inhibition by NBTI, both in isolated hepatocytes and in primary culture. The evidence that the equilibrative transport system is NBTI-insensitive rules out the possibility that NBTI enhances Na⁺-dependent uridine uptake by inhibiting nucleoside efflux from the cells. Indeed, similar effects of NBTI on concentrative nucleoside transport have been reported before, but always in cell types expressing the NBTI-sensitive transport system [1,3,39–41]. Furthermore, in some cases, NBTI addition led only to a much higher intracellular accumulation of the substrate, but without significant modification of the initial uptake rates [3,39], which means that there is a certain delay between effective blocking of nucleoside efflux and further accumulation of nucleosides in the cell. In our hepatocyte preparations the enhancement of Na⁺-dependent uridine uptake triggered by NBTI was extremely rapid, did not require preincubation and was evident at the first incubation time tested (15 s). It is unlikely that NBTI-resistant uptake is an experimental artifact caused either by incomplete removal of Na⁺ from the incubation medium or by the use of low uridine concentrations because, under the same conditions, the human hepatoma cell line HepG2 and the rat hepatoma cell line FAO, which bear a Na⁺-independent agency as major nucleoside transport activity, show high sensitivity to NBTI inhibition. Moreover, the effect of NBTI was observed, although to a

smaller extent, even at high concentrations of uridine (100 μ M). Nevertheless there is a previous observation suggesting NBTI-sensitive transport in rat hepatocytes [23]. We do not have an explanation for this apparent discrepancy, although recently other investigators have failed to detect significant inhibition of nucleoside uptake and metabolism by NBTI in the isolated perfused rat liver [19].

Although the exact mechanism by which NBTI acts is not yet clear, it seems to depend on the transmembrane gradient of Na^+ . Under conditions in which the Na^+ transmembrane electrochemical gradient was depleted (cells incubated in the presence of ouabain and monensin), but with Na^+ -dependent nucleoside transport still significant (approx. 50 % of control values), no stimulatory effect of NBTI was observed. The effect of NBTI on Na^+ -dependent uridine transport was not observed in plasma membrane vesicles from rat liver [29]. This apparent discrepancy can be explained on the basis that partly purified preparations of plasma membrane do not necessarily preserve all the biological functions of membrane proteins, as previously described for the hepatic lactate carrier [42]. Although this is speculative, it is possible that the protein(s) involved in the NBTI-triggered activation of Na^+ -dependent uridine transport lack(s) biological activity in this type of preparation. Nevertheless the important finding, in the light of these results, is that the use of NBTI as inhibitor of nucleoside transport into liver parenchymal cells seems inappropriate and studies based on this technique should now be re-evaluated.

Na^+ -dependent uridine uptake markedly decreased when cells were placed in primary culture. For most preparations this activity was almost negligible. Measurements of transport stability in suspensions of freshly isolated hepatocytes rule out the possibility that the decay of this transport activity starts along with the procedure of hepatocyte isolation. This may be explained either by the fact that this transport system may have complex trophic requirements, not fulfilled in a classical culture medium, or by a probable inhibition of its expression triggered by an unidentified component of the medium.

At present little is known about the hormonal regulation of most nucleoside transport systems. The first evidence of modulation of the hepatic concentrative nucleoside transport activity comes from the observation that it is induced soon after hepatectomy [36]. Short-term and long-term modulations of this transport activity by glucagon and insulin respectively have also been reported [43]. Moreover Nagy [44] has shown that ethanol inhibits nucleoside uptake in primary cultures of rat hepatocytes, but the author did not fully characterize this transport activity and assumed there was no Na^+ -dependent component, which the present study finds not to be true. Isolated liver parenchymal cells might be a useful tool with which to explore the regulation of the Na^+ -dependent nucleoside transport systems. Long-term cultures of rat hepatocytes will be developed to determine which factors are able to up-regulate Na^+ -dependent nucleoside transport in liver parenchymal cells.

In summary, this study shows that liver parenchymal cells express at least two separate nucleoside transport activities, a concentrative Na^+ -dependent one, which is the same as that reported previously in rat liver plasma membrane vesicles, and a Na^+ -independent, equilibrative, NBTI-insensitive one. The physiological implications of these two activities in the hepatocyte require further investigation, but considering that the liver accumulates nucleosides markedly above their blood concentrations, it is probable that the Na^+ -independent agency is involved in the efflux of these compounds, whereas the Na^+ -dependent systems take them up, possibly from the canaliculi, thus guaranteeing an efficient turnover.

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