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Nucleoside transport in human colonic epithelial cell lines: evidence for two Na^+-independent transport systems in T84 and Caco-2 cells[1]

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Abstract

RT-PCR of RNA isolated from monolayers of the human colonic epithelial cell lines T84 and Caco-2 demonstrated the presence of mRNA for the two cloned Na^+-independent equilibrative nucleoside transporters, ENT1 and ENT2, but not for the cloned Na^+-dependent concentrative nucleoside transporters, CNT1 and CNT2. Uptake of [^{3}H]uridine by cell monolayers in balanced Na^+-containing and Na^+-free media confirmed the presence of only Na^+-independent nucleoside transport mechanisms. This uptake was decreased by 70–75% in the presence of 1 μM nitrobenzylthioinosine, a concentration that completely inhibits ENT1, and was completely blocked by the addition of 10 μM dipyridamole, a concentration that inhibits both ENT1 and ENT2. These findings indicate the presence in T84 and Caco-2 cells of two functional Na^+-independent equilibrative nucleoside transporters, ENT1 and ENT2.

Keywords: Nucleoside transport; Colonic epithelial cell; Reverse transcription polymerase chain reaction

1. Introduction

Polarized intestinal epithelial cells have a limited capacity for de novo synthesis of nucleosides [1], and they therefore depend on nucleoside absorption from luminal sources (e.g. dietary) or 'salvage' from the extracellular fluid at the basolateral membrane. Physiological studies have shown that small intestinal epithelial cells have Na^+-dependent nucleoside transporters on the brush border membrane and Na^+-independent nucleoside transporters on the basolateral membrane [2–5]. The coordinated function of these pathways plays a critical role in the absorption of nucleosides and therapeutically used nucleoside analogs across the intestinal epithelium. It has also been suggested that the basolateral equilibrative pathway may be important in modulating the local concentration of adenosine and thereby limiting activation of secretion in T84 cells via the adenosine A2b receptor [6,7].

Two Na^+-dependent concentrative nucleoside transport systems (CNT1, pyrimidine-selective; CNT2, purine-selective) and two Na^+-independent equilibrative transporters that differ in their sensitivity to inhibition by nitrobenzylthioinosine (NBMPR) (ENT1, NBMPR-sensitive; ENT2, NBMPR-resistant) have been cloned and physiologically characterized in the intestine [8–15]. Previous nucleoside transport studies in the Caco-2 human colonic epithelial

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tumor cell line have reached conflicting conclusions. Some authors suggest the presence of CNT1 [16], while others suggest there is another broadly selective Na^+-dependent system on the brush border membrane [17]. In contrast, studies of the T84 human colonic epithelial tumor cell line have not demonstrated any Na^+-dependent nucleoside uptake [7]. However, as in Caco-2 cells, basolateral Na^+-independent nucleoside uptake has been physiologically demonstrated in T84 cells, but it has been suggested that only the NBMPR-sensitive ENT1 is present [6,7].

In the present study, we identify the isoforms of cloned nucleoside transporters in two cultured human intestinal epithelial cell lines, T84 and Caco-2. We demonstrate, by RT-PCR, the presence of mRNA for Na^+-independent NBMPR-sensitive (ENT1) and NBMPR-resistant (ENT2) equilibrative transporters in monolayers of Caco-2 and T84 cells. We further demonstrate that both cell lines exhibit functional NBMPR-sensitive and -resistant uridine uptake in Na^+-free conditions, consistent with the expression of both ENT1 and ENT2 in these cell lines.

2. Materials and methods

2.1. Materials and cell culture

All standard chemicals were purchased from Sigma (St. Louis, MO), Fisher (Pittsburgh, PA), or Gibco BRL (Grand Island, NY). Dipyridamole and NBMPR were from RBI (Natick, MA). Cell culture media and supplements were from Gibco. [5-^{3}H]Uridine (0.81 TBq/mmol) was purchased from ICN Pharmaceuticals (Irvine, CA).

Human colonic epithelial cells, T84 and Caco-2 cells, were obtained from ATCC (Rockville, MD). T84 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) with 10% fetal bovine serum, penicillin-streptomycin (50 000 U/l-50 mg/l), at 37°C with 5% CO_2 and 95% air. Caco-2 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 10 ml/l non-essential amino acids, penicillin-streptomycin (50 000 U/l-50 mg/l), at 37°C with 5% CO_2 and 95% air. For uptake experiments, cells were grown on polycarbonate permeable filters mounted in inserts for 12-well culture plates from Costar (Cambridge, MA). Medium was changed every other day, with all cells fed on the day prior to experiments. All experiments were carried out with cells from passages 24–32 (Caco-2) or 30–40 (T84) at least 10 days post-confluence.

2.2. RNA isolation and RT-PCR

Total RNA from 10 day post-confluent cultures of both cell lines was isolated using a commercial reagent and the manufacturer's modified guanidine isothiocyanate/acid phenol protocol (TRIzol reagent, Gibco). 2 μg of total RNA was annealed with either oligo-$(dT)_{12-18}$ or random hexanucleotides, and first strand cDNA synthesis was carried out with Superscript II RNase H^- Reverse Transcriptase (SuperScript Preamplification System, Gibco BRL). The products of these reactions were used as templates for PCR amplification.

The following primers were designed and synthesized for use in amplification reactions: human CNT1 cDNA (GenBank accession number U62966), nucleotide positions 1598–1622 (sense, 5′-TAGCCTTCTTGATGGGTGTGGCGT-3′) and 2105–2130 (antisense, 5′-GCACAGATCGTGTGGTTGTAAAACCG-3′); human CNT2, based upon a human fetal lung EST sequence (GenBank accession number AA358822), nucleotide positions 51–73 (sense, 5′-GTCCATTGCTCTGTCCACAGTGG-3′) and 329–348 (antisense, 5′-CCAGTGCCCTCTGGGAAATTC-3′); human ENT1 cDNA (GenBank accession number U81375), nucleotide positions 177–197 (sense, 5′-CCATGACAACCAGTCACCAGC-3′) and 1528–1545 (antisense, 5′-ACAATTGCCCGGAACAGG-3′); and human ENT2, based upon the sequence of the human HNP36 cDNA [18] (GenBank accession number X86681), nucleotide positions 32–51 (sense, 5′-CTTTCACCCCAGGCGCATCC-3′) and 1345–1362 (antisense, 5′-AGCAGCGCCTTGAAGAGG-3′). Reactions were carried out in a PE/Applied Biosystems GeneAmp 9700 (Foster City, CA) for 30 cycles (45 s at 94°C, 45 s at 55°C, and 1.5 min at 72°C), followed by 72°C for 10 min.

As a positive control for the amplification of the human nucleoside transporters, human small intesti-

nal total RNA was used as the starting template (Clontech, Palo Alto, CA). PCR products were analyzed on 1.2% agarose gels. Amplification products of the expected sizes were purified from the gel, cloned into the vector pCR 2.1 (Invitrogen, Carlsbad, CA) and subjected to fluorescent sequencing according to the manufacturer's protocols (PE/Applied Biosystems 377 Automated DNA sequencer).

2.3. Uridine uptake

All experiments were carried out in HEPES-buffered Ringer's solution containing (in mM) 135 NaCl, 5 KCl, 3.33 NaH_2PO_4, 0.83 Na_2HPO_4, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, and 5 HEPES, pH 7.4 at 37°C. Na^+-free buffer contained (in mM) 140 *N*-methyl-D-glucamine (NMDG), 5 HEPES, 5 KH_2PO_4, 1.0 $CaCl_2$, 1.0 $MgCl_2$, and 10 glucose, pH 7.4.

Confluent monolayers of cells were washed three times in HEPES-buffered solution, with or without Na^+, followed by 10 min pre-incubation in the same buffer. HEPES-buffered solution containing [^{3}H]uridine (10 μM; 2 μCi/ml) was then added to either the apical or basolateral compartment. The contralateral compartment was balanced with an equal volume of HEPES-buffered solution. Following incubation of 1–4 min, the cell culture inserts were removed and dipped rapidly 10 times in three successive containers of ice-cold phosphate-buffered saline, pH 7.4. Filters were incubated overnight in 1 ml 5% Triton X-100 solution to solubilize the cells, and radioactivity was measured in a Beckman LS7500 liquid scintillation system (Irvine, CA). The protein content of representative monolayers was determined spectrophotometrically using a commercial bicinchoninic acid assay (Pierce Biochemicals, Rockford IL).

For the study of the effects of inhibitors on Na^+-independent transport, monolayers were incubated for 15 min in HEPES-buffered Na^+-free solution in the presence of 1 μM NBMPR or 1 μM NBMPR+10 μM dipyridamole in both apical and basolateral compartments. Three minute basolateral uridine uptake was then measured in the presence of the same concentration of inhibitor(s). To determine passive uptake (not transporter-mediated) monolayers were pre-incubated in 10 μM dipyridamole, and 3 min uptake was then measured in the presence of excess unlabeled uridine (4 mM).

2.4. Statistical analysis

Data are expressed as means ± S.D. for triplicate estimates in representative time-course uptake experiments. For uptake inhibition studies, data are expressed as means ± S.E.M. for three experiments. Student's *t*-test and analysis of variance (ANOVA) were used for paired and multiple variates, respectively. An overall $P < 0.05$ was considered significant.

3. Results

3.1. Identification of nucleoside transporters in T84 and Caco-2 cells by RT-PCR

To identify the isoforms of cloned nucleoside transporters in T84 and Caco-2 cells, we isolated RNA from confluent monolayers of these cell lines and performed RT-PCR. Results of agarose gel electrophoresis of the RT-PCR amplified products are shown in Fig. 1. Positive controls using human small intestine cDNA with the four pairs of nucleoside transporter-specific primers yielded single PCR prod-

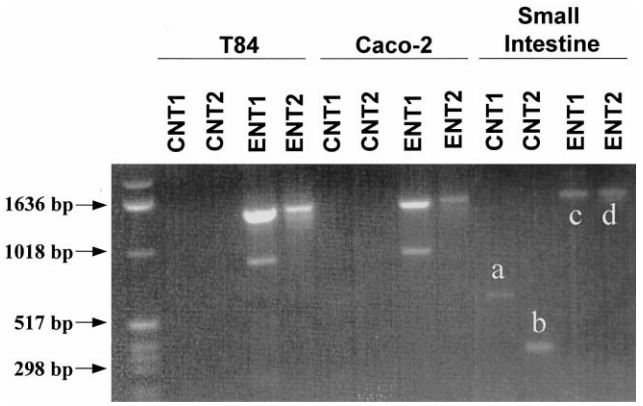

Fig. 1. Agarose gel electrophoresis of RT-PCR amplified products using primers specific for nucleoside transporters in T84 and Caco-2 cells. Fragments are seen only in reactions using oligonucleotide primer pairs for human Na^+-independent nucleoside transporters ENT1 and ENT2. Positive controls with human small intestine total RNA indicate expected sizes of amplified fragments [CNT1 (a), 532 bp; CNT2 (b), 298 bp; ENT1 (c), 1364 bp; ENT2 (d), 1406 bp]. Relevant DNA size markers are indicated on the left.

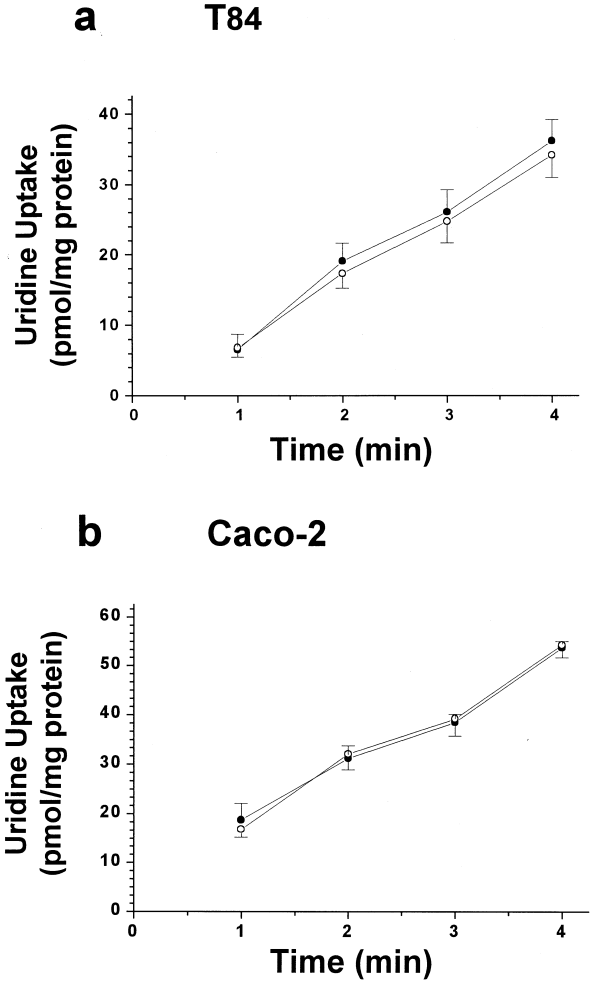

Fig. 2. Time course of basolateral uridine uptake in T84 intestinal epithelial cell monolayers (a) and in Caco-2 intestinal epithelial cell monolayers (b). There is no significant difference in total [^{3}H]uridine uptake (10 μM; 2 μCi/ml) in the presence of Na^+ (filled circles) compared to the absence of Na^+ (open circles). Values are means ± S.D. of triplicate determinations of single representative experiments. Similar results were obtained in three separate experiments.

ucts of the expected sizes. Amplification of cDNA from both T84 and Caco-2 cells demonstrated specific products only when primer pairs specific for either ENT1 or ENT2 were used. No amplified products were observed for CNT1 and CNT2 primers. Sequencing of all cloned PCR products demonstrated 100% identity to the specific GenBank cDNA sequences used for primer design. Sequencing of the smaller amplification product (~900 bp) observed when using the ENT1 primers in T84 and Caco-2 cells yielded a sequence not related to any of the cloned nucleoside transporters.

3.2. Time course of uridine uptake in cell monolayers

Basolateral uridine uptake was measured as a function of time in both the presence and absence of Na^+ (Fig. 2). Uptake in both cell lines was linear over the 4 min period. Therefore subsequent initial rate studies were carried out with a 3 min incubation. There was no significant difference when the uptake was measured in the presence or absence of Na^+ ($P > 0.05$). In contrast, apical uridine uptake, either in the presence or in the absence of Na^+, in both cell lines was statistically indistinguishable from non-mediated passive uptake in the presence of an excess of unlabelled uridine (4 mM), indicating the absence of apical nucleoside transporters in these cell lines (Fig. 3).

3.3. Functional NBMPR-sensitive and NBMPR-resistant uptake in cell monolayers

Basolateral uridine uptake by monolayers of T84 and Caco-2 cells, in the absence of Na^+, were 26.7 ± 2.8 pmol/mg protein/3 min and 45.36 ± 4.1

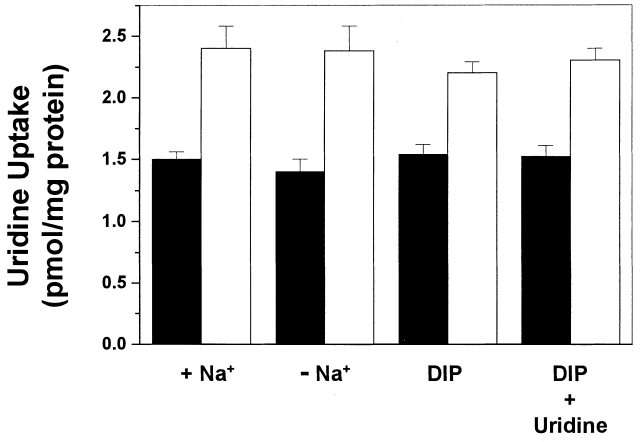

Fig. 3. Apical uridine uptake in T84 intestinal epithelial cell monolayers (solid bars) and in Caco-2 intestinal epithelial cell monolayers (open bars). There is no significant difference in total [^{3}H]uridine uptake (10 μM; 2 μCi/ml; 4 min) in the presence or the absence of Na^+, in the presence of dipyridamole (DIP; 10 μM), or of dipyridamole (10 μM) plus excess unlabelled uridine (uridine; 4 mM). Values are means ± S.D. of triplicate determinations of single representative experiments. Similar results were obtained in three separate experiments.

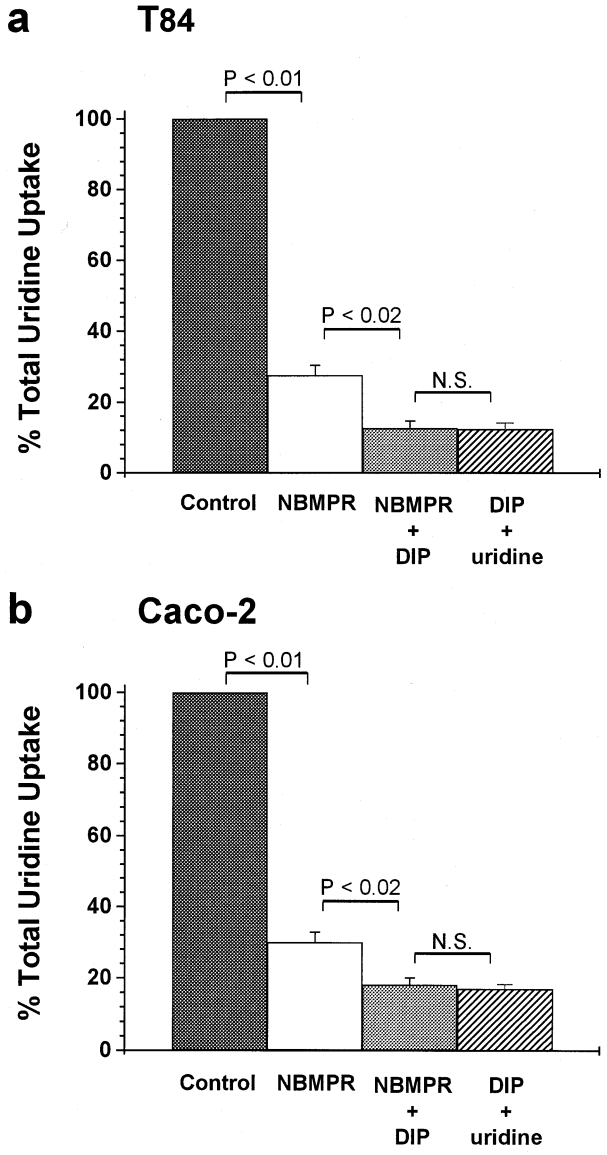

Fig. 4. NBMPR-sensitive and NBMPR-resistant uridine uptake in T84 (a) and Caco-2 cell monolayers (b). [^{3}H]Uridine uptake (10 μM; 2 μCi/ml) was measured, as described in Section 2, in the absence of Na^+ (Control) and in the presence of NBMPR (1 μM), NBMPR (1 μM) plus dipyridamole (DIP; 10 μM), or dipyridamole (10 μM) plus excess unlabelled uridine (uridine; 4 mM). Values are expressed as the percent of total uridine uptake ± S.E.M. ($n = 3$). Uridine uptake not mediated by nucleoside transporters (in the presence of dipyridamole and a 4 mM excess of unlabeled uridine) represents 12.4 ± 1.8% and 17.1 ± 1.2% of total uptake by T84 and Caco-2 cells, respectively. N.S., not significant.

pmol/mg protein/3 min, respectively ($n = 3$). The rate of uridine uptake by T84 cells is comparable to that reported for adenosine in this cell line by Mun et al. [7]. As shown in Fig. 4, NBMPR at 1 μM significantly reduced the total uridine uptake by 72.4 ± 2.8% in T84 cells and by 69.9 ± 2.9% in Caco-2 cells ($P < 0.01$). This concentration of NBMPR is able to completely inhibit uptake mediated by human ENT1 (NBMPR-sensitive; K_i 1–3 nM) in various tissues and cells, including T84 cells [7,12,13]. The addition of 10 μM dipyridamole with the NBMPR, which should additionally block the NBMPR-resistant ENT2-mediated uptake [11,13], resulted in further decreases in uridine uptake of 14.9 ± 2.1% (T84 cells) and 11.9 ± 1.9% (Caco-2 cells) ($P < 0.02$, relative to NBMPR alone). Uridine uptake in the presence of both NBMPR and dipyridamole was not distinguishable from that in the presence of dipyridamole and a large excess of unlabeled uridine (4 mM), in either cell line, indicating that the combination of these compounds totally blocked transporter-mediated nucleoside uptake. The ratios of the contributions of ENT1 (NBMPR-sensitive) relative to ENT2 (NBMPR-resistant, dipyridamole-sensitive) for total uridine uptake in T84 and Caco-2 cells are approximately 5:1 and 6:1, respectively.

4. Discussion

Most mammalian cells transport nucleosides across their membranes by Na^+-independent equilibrative nucleoside transport systems (facilitated diffusion). Epithelial cells from tissues such as small intestine, kidney, lung and liver also possess Na^+-dependent concentrative nucleoside transport processes. These uptake mechanisms are essential for the salvage of extracellular nucleosides, particularly in cells such as intestinal epithelial cells that have a very limited capacity for the de novo synthesis of these compounds.

Recently, two Na^+-dependent concentrative nucleoside transporters and two Na^+-independent equilibrative nucleoside transporters were cloned by expression cloning in oocytes [8,9,14,15]. The two cloned Na^+-dependent nucleoside transporters are termed CNT1, which is pyrimidine-selective [8,14], and CNT2, which is purine-selective [9,15]. The two

Na^+-independent equilibrative nucleoside transporters are termed ENT1, which is sensitive to the inhibitor NBMPR [13], and ENT2, which is resistant to NBMPR [11,19]. NBMPR has no effect on the Na^+-dependent nucleoside transporters. In the present study, we identify the isoforms of cloned nucleoside transporters in two well-characterized cultured human intestinal epithelial cell lines, T84 and Caco-2. T84 cells represent a model of Cl^--secreting intestinal epithelial cells, whereas Caco-2 cells express a combination of absorptive and secretory phenotypes [21]. By RT-PCR, we demonstrated that both T84 and Caco-2 cells lack the messages for CNT1 and CNT2. Using the identical pairs of primers, we were able to amplify CNT1 and CNT2 cDNA fragments from human small intestine (Fig. 1). Functionally, we failed to detect, in both cell lines, any Na^+-dependent uridine uptake activity. Thus, neither Caco-2 cells nor T84 cells express CNT1, CNT2, or other Na^+-dependent nucleoside transport mechanisms.

Our results in T84 cells are consistent with the previous observations by Mun and co-workers that T84 cells lack Na^+-dependent uridine transport [7]. However, in Caco-2 cells, others have reported the presence of Na^+-dependent nucleoside transport. Hu [16] reported that the Na^+-dependent uridine uptake in Caco-2 cells is pyrimidine-selective. Based on the substrate selectivity, this would appear to be CNT1. On the other hand, Belt et al. [17] reported a low level of Na^+-dependent uridine uptake in Caco-2 cells that was broadly selective. Another study by He et al. [20] showed that nucleosides could be transported from the apical surfaces of the Caco-2 cells, although the substrate selectivity and the Na^+ dependence of the transport system were not defined. It is not known what contributes to the discrepancy between the present observation and other previous studies in Caco-2 cells. It might be due to differences in sources, culture conditions, or passages of Caco-2 cells used in the studies [21]. In the present study, we obtained our Caco-2 cells from ATCC, and the cells were used between passages 24 and 32.

The present study demonstrated the presence of ENT1 and ENT2 message in T84 cells and Caco-2 cells. We also demonstrated that both ENT1 and ENT2 are functionally expressed. Although NBMPR-sensitive ENT1 is a major component of Na^+-independent uridine transport in these cells, the NBMPR-resistant ENT2 also contributes significantly ($\sim$15%). Functionally, both ENT1 and ENT2 are restricted to the basolateral membranes, as no apical uridine uptake was observed in either cell line. To our knowledge, this is the first demonstration of ENT1 and ENT2 messages in these intestinal epithelial cells. We are also the first to demonstrate the functional expression of ENT2 in T84 cells. In a previous study, Mun et al. [7] demonstrated that Na^+-independent equilibrative adenosine uptake was confined to the basolateral membrane of T84 cell monolayers. Although they observed that approximately 20% of adenosine transport was not inhibited by a saturating concentration of NBMPR (1 μM; the apparent K_i of inhibition was 1.55 nM), they attributed all Na^+-independent adenosine uptake to the NBMPR-sensitive ENT1 and concluded that the NBMPR-resistant component was due to simple diffusion. In the present study, we found that T84 cells exhibit a similar magnitude (approximately 27.6%) of Na^+-independent uridine uptake that was resistant to inhibition by NBMPR (1 μM). However, this component of uridine uptake could be reduced further to 12.7% by 10 μM dipyridamole (Fig. 4). At this concentration, dipyridamole is able to completely inhibit both ENT1 and ENT2 [11–13]. Inclusion of excess unlabelled uridine (4 mM) with 10 μM dipyridamole did not further inhibit uridine uptake, suggesting that dipyridamole completely inhibited the transporter-mediated uridine uptake in T84 cells. Thus, T84 cells exhibit both functional ENT1 and ENT2. Consistent with our observation, Belt et al. [17] previously reported that Caco-2 cells contain both NBMPR-sensitive and resistant Na^+-independent nucleoside transporters. We further demonstrated that these two nucleoside transport systems are restricted to the basolateral membranes.

As in most cells, the basolateral Na^+-independent nucleoside transporters in Caco-2 cells and T84 cells are believed to be important in the salvage of extracellular nucleosides, in particular adenosine that is produced and released during normal cellular metabolism. Furthermore, in T84 cells, the basolateral Na^+-independent nucleoside transporters are involved in maintaining the extracellular concentrations of adenosine below the threshold of activation

of the adenosine A2b receptor, the activation of which triggers chloride secretion [6,7,23]. Thus, such a nucleoside salvage mechanism prevents excessive adenosine-elicited chloride secretion under basal conditions in T84 cells. In light of the fact that no Na^+-dependent nucleoside transport could be demonstrated in Caco-2 cells, the use of this cell line as a model for human small intestinal epithelial cells in the study of trans-epithelial nucleoside transport appears to be limited. However, transfection of cloned human Na^+-dependent nucleoside transporters into the cells would potentially generate such models. For instance, the Na^+-dependent glucose transporter SGLT1, which is expressed at low levels, has been transfected and expressed in the apical domain of Caco-2 cells, permitting studies of the biology of this transporter [22].

In conclusion, the T84 and Caco-2 cell lines express two types of Na^+-independent equilibrative nucleoside transport systems, the NBMPR-sensitive ENT1 and NBMPR-resistant ENT2. Although ENT2 contributes 15% and 17% of total Na^+-independent uridine uptake in Caco-2 and T84 cells, respectively, this component of uridine uptake is of physiological importance. For instance, treating T84 cells with the cytotoxic nucleoside tubercidin in the presence of 1 μM NBMPR, which allows the tubercidin to enter only through the NBMPR-resistant ENT2 system, results in cell death (Ward and Tse, unpublished observation). Since both ENT1 and ENT2 systems are physiologically active, we suggest that both ENT1 and ENT2 are physiologically important in salvaging nucleosides under basal conditions. Perhaps, for fine-tuning the extracellular nucleoside concentrations, these two transport systems might be differentially regulated under various physiological conditions. To this end, the NBMPR-sensitive ENT1 system in T84 cells has been shown to be regulated by phorbol esters, but not by cAMP and cellular ATP depletion [7].

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