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Passive and carrier-mediated permeation of different nucleosides through the reconstituted nucleoside transporter

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When reconstituted into proteoliposomes, the human erythrocyte nucleoside transporter catalysed nitrobenzylthioguanosine (NBTGR)-sensitive zero-*trans* influx of three different nucleosides at broadly similar rates (inosine, uridine > adenosine). However, proteoliposomes also exhibited high rates of NBTGR-insensitive uptake of adenosine, making this nucleoside unsuitable for reconstitution studies. Equivalent high rates of adenosine influx were observed in protein-free liposomes, establishing that this permeability pathway represents simple diffusion of nucleoside across the lipid bilayer. In contrast to adenosine, inosine and uridine exhibited acceptable rates of NBTGR-insensitive uptake. Of the two, inosine is the more attractive permeant for reconstitution experiments, having a 2.5-fold lower basal membrane permeability. Studies of nucleoside transport specificity in reconstituted membrane vesicles should take account of the widely different passive permeabilities of different nucleosides.

Nucleoside transport across the human erythrocyte membrane occurs by a facilitated diffusion process which can be inhibited by nanomolar concentrations of NBMPR and NBTGR [1–3]. The specificity of the system in intact cells is sufficiently broad that both physiological nucleosides (purine or pyrimidine ribosides and deoxyribosides) and a wide variety of nucleoside analogues are accepted as substrates. Photoaffinity labelling and reversible ligand binding experiments using [^{3}H]NBMPR [4–7] have identified the human and pig erythrocyte nucleoside transporters as band 4.5 polypeptides (nomenclature of Steck [8]) and isolated band 4.5 polypeptides from the two species have been shown to catalyse NBTGR-sensitive uridine transport when reconstituted into proteoliposomes [7,9]. NBTGR-sensitive nucleoside transporters from other sources, including guinea-pig lung, heart and brain, have broadly similar molecular weights to that of the human erythrocyte system (apparent M_r (av) 55000) [10–14]. Recently, the human erythrocyte nucleoside transporter has been purified to apparent homogeneity by a combination of ion-exchange and immunoaffinity chromatography [15].

In previous reconstitution experiments using crude erythrocyte membrane extracts and purified band 4.5 polypeptides [7,9,15], uridine was chosen as the nucleoside permeant because its transport into mammalian cells has been studied extensively [16–19]. Kinetic parameters obtained in the reconstituted system could therefore be compared directly with published values for intact cells. However, other nucleosides, particularly adenosine and inosine, can be considered of greater physiological interest. For example, adenosine functions as a novel form of cellular regulator [20], as an ATP precursor [21,22] and as an endogenous vasodilator [23] whereas inosine is an *in vivo* energy source for adult pig erythrocytes, cells which are unable to metabolise glucose [24–26]. Therefore, in the present study we compared the abilities of adenosine, inosine and uridine to serve as permeants in the reconstituted system.

Blood from healthy human volunteers was collected into heparin and the erythrocyte membranes harvested and depleted of extrinsic proteins as described previously [6]. These membranes (2 mg protein/ml) were solubilised at 4°C by suspension in 46 mM *n*-octyl glucoside, 50 mM Tris-HCl, 2 mM dithiothreitol (pH 7.4) with stirring for 30 min, after which the preparation was centrifuged at 130000 × *g* for 1 h [6,27]. The mem-

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Abbreviations: NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine.

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brane-free supernatant was collected and detergent removed by dialysis against four changes of 10 mM Tris, 0.2 mM dithiothreitol (pH 7.4 at 4°C) over a period of 48 h. This solubilised crude membrane extract was reconstituted into soybean phospholipid vesicles by freeze-thaw-sonication as described previously [9]. Nucleoside zero-*trans* influx into the reconstituted proteoliposomes was measured at 15°C by a centrifugal gel filtration method using a permeant concentration of 50 μM [9]. At this temperature, the apparent K_m value for zero-*trans* influx of uridine in the reconstituted system is 0.21 mM [9]. Briefly, columns of Sephadex G-50 (fine), which had been pre-equilibrated with 20 μM NBTGR, 10 mM Tris-HCl, were poured to the 1 ml mark in disposable 1 ml (tuberculin) syringes. The columns were centrifuged at $200 \times g$ for 2 min in the swinging bucket rotor of a bench centrifuge shortly before use. The prepared columns were kept on ice. All subsequent procedures were carried out in a 4°C cold room. Incubations were initiated by adding 45 μl of reconstituted vesicles ($\pm$20 μM NBTGR) to 45 μl of ^{14}C-labelled nucleoside (2 μCi/ml) in the same 10 mM Tris-HCl buffer. Uptake was terminated by the rapid addition of 20 μl of an ice-cold stopping solution containing 20 μM NBTGR in Tris buffer, and a 75 μl sample of the reaction mixture was immediately applied to a centrifuged column. After the sample entered the gel, 20 μl of stopping solution was added to the syringe column which was then recentrifuged as described above, and the eluate was collected directly into a scintillation minivial in the centrifuge bucket. Radioactivity present in the eluate was measured by liquid scintillation spectrometry with appropriate quench and background correction. Blank values for uptake assays were determined by centrifugal gel column processing of samples taken immediately after mixing ice-cold NBTGR-treated vesicles and ice-cold ^{14}C-labelled nucleoside. These blanks were subtracted from measurements of uridine uptake by reconstituted vesicles. Protein was determined according to Lowry et al. [28] and phospholipid by phosphate analysis [29].

Fig. 1 compares representative time courses of uridine, inosine and adenosine uptake ($\pm$20 μM NBTGR) into vesicles reconstituted with *n*-octyl glucoside-solubilised crude membrane extract. In two such experiments, uptake of adenosine was 517(557, 475) pmol/mg protein in 10 s, compared with values of 319(362, 275) and 336(379, 292) pmol/mg protein in 10 s for inosine and uridine, respectively. The observed high uptake rate for adenosine did not, however, reflect transporter preference for this nucleoside, inosine, uridine and adenosine giving broadly similar rates of carrier-mediated (NBTGR-sensitive) transport into the reconstituted vesicles (293(335, 250), 273(300, 245) and 206(236, 175) pmol/mg protein in 10 s, respectively). Instead, it was striking that the magnitudes of

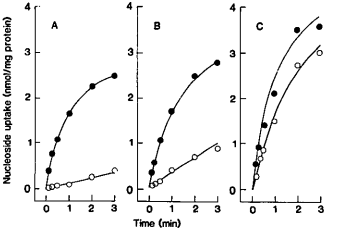

Fig. 1. Time courses of inosine, uridine and adenosine uptake into vesicles reconstituted with crude human erythrocyte membrane extract. Inosine (Panel A), uridine (Panel B) and adenosine (Panel C) uptake (50 μM) were measured at 15°C in the presence (open symbols) and in the absence (closed symbols) of 20 μM NBTGR by centrifugal gel filtration as detailed in the text. Each assay contained 11 μg of protein and 0.52 μmol of lipid. Values are means of duplicate estimates.

NBTGR-insensitive uptake differed considerably for the three nucleosides. For the same two experiments, rates of NBTGR-insensitive nucleoside uptake into the reconstituted vesicles were 26(27, 25), 63(79, 47) and 311(321, 300) pmol/mg protein in 10 s for inosine, uridine and adenosine, respectively. These values are equivalent to 3.3, 8.1 and 39.8 pmol/μmol phospholipid per min, respectively. Thus, the NBTGR-insensitive components of nucleoside uptake were in the ratio of 1 : 2.5 : 12.1 for inosine, uridine and adenosine. In agreement with previous control experiments with uridine [7,9], exogenous lipid and membrane extract were both essential for reconstitution of NBTGR-sensitive inosine and adenosine transport activity (data not shown).

It was considered likely that NBTGR-insensitive nucleoside uptake represented simple diffusion of permeant across the vesicle membrane. To test this possibility, the permeability of the phospholipid bilayer to the three nucleosides was measured directly. Fig. 2 shows time courses of uridine, inosine and adenosine uptake measured in vesicles prepared in the absence of protein. Consistent with the results obtained for reconstituted vesicles, estimated initial rates of nucleoside permeation into protein-free liposomes (two experiments) were 3.5(3.7, 3.2), 8.9(10.1, 7.6) and 44.6(52.3, 36.9) pmol/μmol phospholipid per min for inosine, uridine and adenosine, respectively, giving ratios of 1 : 2.5 : 12.7 with respect to inosine. The magnitudes of these fluxes for the different nucleosides correlates well with their respective octanol/water partition coefficients [30] and also with the relative permeabilities of nucleoside transport-deficient sheep erythrocytes to these three nucleosides [21]. As expected, nucleoside

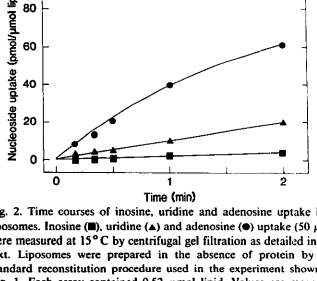

Fig. 2. Time courses of inosine, uridine and adenosine uptake into liposomes. Inosine (■), uridine (▲) and adenosine (●) uptake (50 μM) were measured at 15°C by centrifugal gel filtration as detailed in the text. Liposomes were prepared in the absence of protein by the standard reconstitution procedure used in the experiment shown in Fig. 1. Each assay contained 0.52 μmol lipid. Values are means of duplicate estimates.

uptake into protein-free liposomes was not affected by NBTGR (data not shown).

We conclude from the present results that the solubilised human erythrocyte nucleoside transporter is able to catalyse NBTGR-sensitive influx of inosine and adenosine in addition to uridine transport when reconstituted into phospholipid vesicles. We have previously shown that uridine transport into reconstituted vesicles is inhibited by adenosine and inosine [9]. Taken together, these observations demonstrate that the reconstituted nucleoside transporter exhibits broad specificity for both purine and pyrimidine nucleosides, a finding consistent with data from intact cells [1–3]. The present results also demonstrate that liposomes are selectively permeable to adenosine compared with uridine and inosine, adenosine exhibiting a high diffusion permeability across the lipid bilayer. Physiologically, this means that cells will be significantly permeable to adenosine, even in the absence of a nucleoside carrier. As an example of this, we have demonstrated previously that both nucleoside transport-positive and nucleoside transport-deficient sheep erythrocytes have the ability to synthesise ATP when incubated in the presence of extracellular adenosine and a suitable energy source, e.g., glucose [21]. Diffusion across the lipid bilayer is the primary route by which the antiviral dideoxynucleosides 3′-azido-3′-deoxythymidine (AZT) and 3′-dideoxythymidine enter cells [31,32].

Unfortunately, the high basal lipid permeability of adenosine renders it unsuitable for reconstitution studies. Even in the present series of experiments with human erythrocyte membranes (which have a high density of nucleoside transporters), the ratio of mediated-to-nonspecific uptake of adenosine was only 0.7 compared with 4.3 for uridine and 11.3 for inosine. In other experiments, we have been unable to demonstrate carrier-mediated adenosine transport in vesicles reconstituted with *n*-octyl glucoside extracts of purified guinea-pig lung plasma membranes. These membranes exhibit a 3-fold lower specific activity of NBMPR-binding than human erythrocyte protein-depleted membranes (Tse, C.M., Shi, M.M. and Young, J.D., unpublished results). In contrast, we can detect routinely NBTGR- and adenosine-sensitive uridine transport into such vesicles. Since inosine has a 2.5-fold lower basal permeability than uridine, we anticipate that inosine may prove to be an even better permeant for reconstitution experiments, particularly in situations where the membrane density of NBMPR-binding sites is low.

Relative rates of passive and carrier-mediated transport of nucleosides are substantially lower in intact human and nucleoside transport-positive sheep erythrocytes than in reconstituted liposomes [1–3,21,30]. This is largely attributable to the low protein : lipid and high surface area/volume ratios of reconstituted liposome preparations. In kinetic studies to be published in detail elsewhere, we have also established that the mobilities for the loaded and empty (unloaded) carrier in the reconstituted system are both reduced compared with intact cells, the mobility of the empty carrier being more substantially impaired. As a consequence, the reconstituted human erythrocyte nucleoside transporter in asolectin vesicles exhibits 6–7% activity relative to intact erythrocytes when assayed under zero-*trans* efflux and influx conditions, and 18% when assayed under equilibrium exchange influx conditions. The altered kinetic properties of the reconstituted nucleoside transport system are likely to reflect the change of transporter lipid environment. Future research into nucleoside transporter reconstitution should therefore also focus on systematic evaluation of the effects of vesicle lipid composition on carrier-mediated (and basal) nucleoside permeation. In preliminary experiments, using uridine as permeant, we have found that reconstituted vesicles prepared from extracted human erythrocyte lipids give an improved mediated/basal uptake ratio when compared with asolectin liposomes. Both carrier-mediated and passive uridine permeability were affected under these conditions.

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