A Nucleoside Transporter Is Functionally Linked to Ectonucleotidases in Rat Liver Canalicular Membrane*

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Prevention of nucleoside loss in bile is physiologically desirable because hepatocytes are the main source of nucleosides for animal cells which lack de novo nucleoside biosynthesis. We have demonstrated a Na⁺-gradient-energized, concentrative nucleoside transport system in canalicular membrane vesicles (CMV) from rat liver by studying [³H]adenosine uptake using a rapid filtration technique. The Na⁺-dependent nucleoside transporter accepts purine, analogues of purine nucleosides and uridine; exhibits high affinity for adenosine (apparent Kₘ = 14 μM); is not inhibited by nitrobenzylthioinosine or dipryridamole, and is present in CMV but not in rat liver sinusoidal membrane vesicles. Adenosine transport in right side-out CMV was substantially greater than with inside-out CMV. CMV also contain abundant ecto-ATPase and ecto-AMPase (5′-nucleotidase). These ectoenzymes were shown to degrade nucleosides into nucleosides which were conserved by the Na⁺-dependent nucleoside transport system.

The movement of nucleosides across animal cell plasma membranes is mediated by several transport systems (1). There are two types of facilitated nucleoside transport which differ in their sensitivity to the inhibitor, nitrobenzylthioinosine (NBTI). One type is inhibited by low concentrations of NBTI (IC₅₀ = 1-10 nM); whereas, the IC₅₀ of the other type is 5-10 μM. Both are completely inhibited by dipryridamole (DPA) and papaverine (2). In addition, there are two distinct Na⁺-dependent nucleoside transport systems which differ in their substrate specificities. One transport system primarily accepts purine nucleosides, and the other primarily accepts pyrimidine nucleosides. Uridine and adenosine can be accepted by either (3-6).

Extracellular nucleosides are either released from cells or generated from nucleotide breakdown by ectonucleotidases. In hepatocytes, 5′-nucleotidase and Ca²⁺,Mg²⁺-ecto-ATPase are primarily localized in the canalicular domain of the plasma membrane (7). The function of these two ectonucleotidases is postulated to be hydrolysis of extracellular nucleotides and formation of nucleosides which may be conserved by hepatocytes through the action of nucleoside transporters.

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The abbreviations used are: NBTI, nitrobenzylthioinosine; Ara-C, cytosine arabinoside; AZT, azidothymidine; ara-A, vidarabine; γ-GT, γ-glutamyltransferase; CMV, canalicular membrane vesicle; SMV, sinusoidal membrane vesicle; HPLC, high-performance liquid chromatography; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; DPA, dipryridamole.
Nucleoside Transport and Ecto-ATPases

Results

Adenosine Transport in CMV and SMV—In the presence of an initial 80 mM NaCl gradient (out > in), a transient overshoot of CMV-associated adenosine above its equilibrium value was observed. CMV-associated adenosine reached a maximum at 10 s and decreased thereafter. In a 80 mM KCl gradient (out > in), no overshoot was observed (Fig. 1A).

CMV-associated adenosine in the presence of the Na+ gradient decreased significantly when the osmolarity was increased (Fig. 1B). The values obtained from extrapolation to infinite high osmolarity indicate that 64% of CMV-associated adenosine represents specific uptake into the intravesicular space. Na+ gradient-dependent adenosine uptake was abolished when the transport experiment was performed on ice (data not shown).

When anion substitution was used to alter the membrane potential, the effects on Na+-stimulated adenosine uptake were as shown in Fig. 1A. Uptake was greatest in the presence of the permeant lipophilic anion, SCN−, and least with the relatively impermeant anion, SO42−. Alternatively, Na2SO4 cotransporter may collapse the Na+ gradient and thereby reduce adenosine uptake. These results suggest that the transmembrane electrochemical Na+ gradient is the source of energy necessary for Na+-dependent adenosine transport.

When the extravesicular Na+ concentration was increased from 2.5 to 160 mM, the rate of adenosine uptake increased until saturation occurred at NaCl concentrations above 80 mM (Fig. 2). The hyperbolic uptake of adenosine in relation to NaCl concentration conformed to simple saturation kinetics consistent with a single Na+-binding site on the transporters. There was no effect of KCl concentration on adenosine uptake. When the transmembrane Na+ gradient was dissipated by adding monensin, a cation ionophore, adenosine uptake was significantly decreased (data not shown). These results indicate that concentrative transport of adenosine in CMV is Na+-dependent.

Na+-dependent adenosine uptake was also dependent on the adenosine concentration (Fig. 3A). Uptake values followed simple Michaelis-Menten kinetics with an apparent Km of 14 μM and Vmax of 142 pmol/mg of protein/s (Fig. 3B).

In contrast to results observed with CMV, adenosine uptake into SMV was not increased by a Na+ gradient (data not shown).

Effect of Nucleosides and Nucleotides on Adenosine Uptake—To explore the substrate specificity of the transport system in CMV, several purines and pyrimidines were used in inhibition studies. Inosine, guanosine, and uridine were strong inhibitors, whereas cytidine and thymidine inhibited adenosine uptake only at high concentrations (Table I).

The Na+-dependent adenosine uptake in CMV was inhibited in the presence of 2 mM ATP (Fig. 4). To investigate whether nucleotides or their metabolites are the direct inhibitors, the effect of different nucleotides on initial adenosine uptake (2 s) was examined. In the presence of a high concentration (1 mM) of unlabeled adenosine, 1 μM [3H]adenosine uptake was almost completely abolished, whereas, at the same concentrations of AMP, ADP, and ATP, uptake was 20, 66, and 79% of control, respectively. The nonhydrolyzable ATP analogue (AMP-PNP) did not inhibit the transport system. Similarly, 1 mM GTP, UTP, and CTP had little effect. In the presence of 100 μM unlabeled adenosine or inosine, 80–90% of adenosine (1 μM) uptake was inhibited, whereas, at the same concentrations of AMP or IMP, less than 50% of adenosine (1 μM) uptake was inhibited (Table I). These results suggest that the transporter is nucleoside-specific.

Although nucleotides are not direct substrates for the transport system, they can be degraded to nucleosides by ectonucleotidases in CMV. As shown in Fig. 5, when CMV was incubated with 1 mM ATP, ATP was completely hydrolyzed in 1 min and adenosine and inosine concentrations increased with incubation time. In excess GTP (10 × of ATP), ATP breakdown and production of nucleosides were inhibited. At 1 min, 79% of ATP remained and no nucleosides were detected.
**Nucleoside Transport and Ecto-ATPases**

Fig. 3. Effect of adenosine concentration on Na*-dependent adenosine uptake in CMV. A, adenosine uptake was determined at 2 s in the presence of a Na* gradient (80 mM NaCl outside; 0 mM NaCl inside (open circle)); or in the presence of K* gradient (open triangle). Na*-dependent adenosine uptake (solid circle) was obtained by subtracting the bottom line from the top curve. B, reciprocal plot of Na*-dependent adenosine uptake at 2 s for adenosine concentrations between 0.375 and 12 µM. The values shown are the mean of three experiments.

![Graph](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Nucleotides or nucleosides</th>
<th>Relative uptake</th>
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<tbody>
<tr>
<td>Control</td>
<td>100%</td>
</tr>
<tr>
<td>Guanosine</td>
<td>28.2 ± 7.4</td>
</tr>
<tr>
<td>Uridine</td>
<td>29.0 ± 0.7</td>
</tr>
<tr>
<td>Cytidine</td>
<td>97.7 ± 0.3</td>
</tr>
<tr>
<td>Thymidine</td>
<td>101.5 ± 3.1</td>
</tr>
<tr>
<td>Inosine</td>
<td>8.5 ± 1.7</td>
</tr>
<tr>
<td>IMP</td>
<td>52.8 ± 3.2</td>
</tr>
<tr>
<td>Adenosine</td>
<td>17.8 ± 3.6</td>
</tr>
<tr>
<td>AMP</td>
<td>54.4 ± 7.3</td>
</tr>
<tr>
<td>ADP</td>
<td>ND</td>
</tr>
<tr>
<td>ATP</td>
<td>ND</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>ND</td>
</tr>
<tr>
<td>GTP</td>
<td>ND</td>
</tr>
<tr>
<td>UTP</td>
<td>ND</td>
</tr>
<tr>
<td>CTP</td>
<td>ND</td>
</tr>
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</table>

Relative uptake (% of total) at 1 min with or without 10 mM GTP.

*The results are presented as average of two experiments.

**FIG. 5.** Time course of ATP metabolism in CMV. CMV (60–105 µg of proteins) were incubated with 1 mM ATP at 20 °C and ATP metabolites were analyzed by HPLC. Solid square, ATP; open square, total di- and monophosphate nucleotides which include ADP, AMP, and IMP; solid circle, nucleosides which include adenosine and inosine. Inset, 1 mM ATP metabolism products (% of total) at 1 min with or without 10 mM GTP.

Fig. 6. Time course of [3H]ATP metabolites uptake in CMV. Uptake of ATP metabolites was measured by incubating CMV with 1 µM [3H]ATP in the presence of 80 mM NaCl (solid circle) or 80 mM KCl (open circle).

**FIG. 4.** Effect of ATP on Na*-dependent adenosine uptake. Uptake of 1 nM [3H]adenosine was determined in the presence (solid circle) or absence (open circle) of 2 mM ATP in incubation medium. Uptake was calculated by subtracting values obtained in the presence of 80 mM KCl from the presence of 80 mM NaCl.

Further evidence that nucleosides which are derived from ATP hydrolysis can be transported into CMV by a Na*-dependent adenosine transport system is provided in Fig. 6. By using [2,8-3H]ATP instead of [2,8-3H]adenosine, a similar Na*-dependent overshoot was observed in CMV and was completely inhibited by 1 mM unlabeled adenosine or 2 mM GTP (data not shown). This result further indicates that both ectonucleotidases and Na*-dependent nucleoside transport system are necessary for nucleoside conservation.

**Effect of Nucleoside Transporter Inhibitors—**The nonhydrolyzable inosine analogue, formycin B, inhibits the purine-specific, Na*-dependent transport system (3). With 200 µM formycin B, adenosine transport activity in CMV decreased to 18% of control. With the same concentration of Ara-C, Ara-A, and AZT, transport activity was 61, 17, and 66% of control, respectively (Table II). NBTI and DPA are specific inhibitors of facilitated nucleoside transport (1, 2). Na*-dependent nucleoside transport in CMV was not inhibited by 10 µM NBTI or 200 µM DPA (Table II, Fig. 7). Na*-dependent overshoot of adenosine uptake was increased and prolonged in the presence of NBTI or DPA, which probably results from inhibition of nucleoside efflux.

**Functional Asymmetry of Na*-dependent Nucleoside Transport in CMV—**CMV obtained by the procedure described under "Experimental Procedures" are approximately 80–90% right side-out and 10–20% inside-out. To test whether, like the Na*+-glucose cotransporter (12), the Na*-dependent nucleoside transport is functionally asymmetric, inside-out and
The characteristics of nucleoside transport systems in erythrocytes and other cells in culture have been studied extensively (1, 2, 13-16). Almost all animal cells possess a low affinity, high capacity, and nonconcentrative nucleoside transporter which has wide substrate specificity (1, 17). Na+-dependent and concentrative nucleoside transport has been characterized in epithelial cells from intestine and kidney (4, 6, 18, 19, 20) and in macrophage cells (21). The cloning of a nucleoside transporter has not been reported.

The importance of bile canalicular Na+-dependent nucleoside transport system is related to the following unique functions of liver: first, some animal cells, such as bone marrow cells, certain brain cells, intestinal mucosa cells, leukocytes, and erythrocytes, are deficient in de novo purine biosynthesis and depend on preformed purines, which are mainly derived from the liver and from dietary sources (1, 22, 23). Therefore, a concentrative nucleoside transport system is physiologically desirable for maintaining the nucleoside concentration in hepatocytes and preventing nucleoside loss in bile. Second, extracellular ATP profoundly damages hepatocytes and experimentally produces cholestasis (24). The abundant bile canalicular ecto-Ca²⁺,Mg²⁺ATPase and 5'-nucleotidase rapidly degrade ATP to adenosine (7, 25). The dual processes of nucleotide breakdown and nucleoside conservation are important physiologically and pathologically.

If the function of nucleoside transport is conservation, the direction of this transport would be postulated to be from outside to inside. A functional asymmetry has been found in Na+-d-glucose cotransporter (12). Our studies which compare transport activity of the nucleoside transport in separated inside-out and right side-out vesicles provide evidence that the Na+-dependent nucleoside transporter is also asymmetric, with main functional direction from outside into inside of hepatocytes. Moreover, there is a Na⁺ gradient across the canalicular plasma membrane (144–161 mM in hepatic bile compared to 12 mM in hepatocytes) which determines the unidirectional function of the Na+-dependent nucleoside transport system.

Guanosine, inosine, uridine, and formycin B are good inhibitors of cytidine and thymidine are relatively poor inhibitors of Na+-dependent adenosine uptake in CMV. These results suggest that the Na+-dependent nucleoside transport system in CMV is functionally similar to the Na+-dependent purine specific nucleoside transport in other tissues (6, 18).

Many antiviral compounds are purine and pyrimidine nucleoside analogues which inhibit viral DNA synthesis. Ara-C and AZT, two pyrimidine nucleoside analogues, partially inhibited nucleoside transport activity, and Ara-A, a purine nucleoside analogue, strongly inhibited nucleoside transport activity which may be related to the clinical toxicity of these compounds.

Ecto-ATPases are present in many tissues. Unlike transport ATPases, which transport ions across membranes and use intracellular ATP as an energy source, the ecto-ATPases have an ATP-hydrolyzing site localized on the outside of the cell surface. Hepatocellular ecto-ATPase is enriched in the canalicular domain, has broad nucleotide-hydrolyzing activity, and can hydrolyze ATP, GTP, UTP, CTP, ADP, and GDP to a similar extent (7, 26, 27). By analyzing ATP metabolites and nucleoside transport activity in CMV, we observed a functional link between nucleoside transport and ectonucleotidases. Coupling of these two systems in CMV is unknown due to technical problems in sampling...

### TABLE II

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentrations</th>
<th>Relative uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Formycin B</td>
<td>18.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Ara-C</td>
<td>61.0 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>66.0 ± 8.0</td>
<td></td>
</tr>
<tr>
<td>Ara-A</td>
<td>17.7 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>NBTI</td>
<td>174.1 ± 9.7</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 7. Effect of DPA on adenosine uptake in CMV.** The incubation medium contained 1 μM [³H]adenosine, 80 mM NaCl (circle) or 80 mM KCl (square) with (solid symbols) or without (open symbols) 200 μM DPA.

**FIG. 8. Effect of adenosine concentration on Na⁺-dependent adenosine uptake in inside-out and right side-out vesicles.** Na⁺-dependent adenosine uptake at 2 s was calculated by subtracting values obtained in the presence of a K⁺ gradient from in the presence of a Na⁺ gradient (80 mM outside; 0 mM inside). The results represent the average of duplicate determinations. Solid circles, data from right side-out vesicles; open circles, data from inside-out vesicles.

The characteristics of nucleoside transport systems in erythrocytes and other cells in culture have been studied...
The hepatocyte is the likely source of canalicular ATP. There is substantial vesicular transport from the sinusoidal plasma membrane (transcytosis), Golgi and endoplasmic reticulum; these vesicles fuse with the bile canalculus and may, thereby, facilitate constant release of ATP into the bile canalculus. Hypoxia produces cholestasis in the liver (24) and releases ATP from human erythrocytes and cardiac myocytes (30, 31). These data permit the following hypothesis. Small amounts of ATP are normally released from hepatocytes into the bile canaliculi, how ATP is released from cells, what concentrations of ATP are required to produce cholestasis associated with increasing the concentration of intracellular Ca\(^{2+}\) (24). To validate this hypothesis, we are performing studies to determine whether ATP is normally present in the bile canaliculi, how ATP is released from cells, what concentrations of ATP are required to produce cholestasis, and whether the P\(_{2y}\) class purine receptor (32) in the canaliculus membrane is responsible for signal transduction in ATP-induced cholestasis.

While this work was in review, a Na\(^+\)-dependent purine transport system was also demonstrated in rat canalicular membrane vesicles (33).

REFERENCES