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MOLECULAR PHARMACOLOGY, 50:1529–1535 (1996).

Transport of Adenosine by Recombinant Purine- and Pyrimidine-Selective Sodium/Nucleoside Cotransporters from Rat Jejunum Expressed in *Xenopus laevis* Oocytes

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Received April 2, 1996; Accepted August 5, 1996

SUMMARY

Two major Na^+-dependent nucleoside transporter subtypes implicated in adenosine transport in mammalian cells are distinguished functionally on the basis of substrate specificity: one is selective for pyrimidine nucleosides but also binds adenosine, and the other has selectivity for purine nucleosides but also binds uridine. Transportability of adenosine by the purine-selective system has been established by measurements of [^{3}H]adenosine fluxes, whereas the conclusion that adenosine is permeant of the pyrimidine-selective system is based on inhibition assays. We investigated adenosine transport mediated by a recombinant pyrimidine-selective rat jejunal/kidney Na^+/nucleoside cotransporter (rCNT1) expressed in *Xenopus laevis* oocytes and compared it with that mediated by a recombinant purine-selective rat jejunal/liver Na^+/nucleoside cotransporter (rCNT2). Adenosine fluxes mediated by rCNT1 were 1 order of magnitude lower than those mediated by rCNT2. In kinetic studies, rCNT1 transported adenosine with an apparent K_m value for influx (26 μM) similar to that for uridine but with a very much lower V_{max} value, and the V_{max}/K_m ratios were 0.003 and 0.57 for adenosine and uridine, respectively. Recombinant rCNT1 mediated efflux of [^{3}H]uridine from preloaded oocytes, demonstrating a capacity for bidirectional transport of nucleoside permeants. Uridine efflux was stimulated by extracellular uridine and inhibited by extracellular adenosine, suggesting that the rate of conversion of rCNT1 from its outward-facing conformation to its inward-facing conformation was increased when the transporter was complexed with uridine and decreased when it was complexed with adenosine. Thus, although rCNT1 binds adenosine and uridine with similar affinities, it kinetically favors transport of uridine.

Adenosine has a special physiological role as a local signaling molecule with regulatory functions in lipolysis, neurotransmitter release, platelet aggregation, coronary vasodilation, and renal vasoconstriction (1, 2). Adenosine is also a precursor of important energy-rich cellular metabolites. Plasma membrane transport of adenosine is mediated by both equilibrative and concentrative nucleoside transporter proteins. Equilibrative nucleoside transporters are widely distributed in mammalian cells and tissues and can be divided into two classes (*es* and *ei*) depending on whether they are sensitive (*es*) or insensitive (*ei*) to inhibition by nitrobenzylthioinosine (3). Active, Na^+-linked nucleoside transporters were first detected in intestinal (4–7) and renal epithelia (8–13) but are also expressed in many different mammalian tissues and cell types, including choroid plexus (14), liver (15), splenocytes (16), macrophages (17), and murine (18–22) and human leukemic cells (23, 24).

Two major classes of Na^+-dependent nucleoside transporters implicated in adenosine transport in mammalian cells have complementary, but overlapping, specificities for purine and pyrimidine nucleosides (1). Nucleoside transporters designated N1 or *cif* are selective for purine nucleosides and uridine (4, 7, 8, 11, 13, 15–22, 25). Nucleoside transporters designated N2 or *cit* are selective for pyrimidine nucleosides and adenosine (4, 12, 13, 26, 27). A third class of nucleoside transporters designated N3 or *cib* has the ability to transport a wide range of both purine and pyrimidine nucleosides (14, 24, 25, 28), and a human kidney N2/*cit*-type process with selectivity for pyrimidine nucleosides, adenosine, and guanosine has been given the designation N4 (29), although it otherwise exhibits the characteristics of N2/*cit*.

Transportability of adenosine by the N1/*cif* and N3/*cib* processes of rabbit intestinal and rat renal brush-border membrane vesicles (6, 8, 10, 11), rat hepatocytes (15), murine leukemia L1210 cells (20, 21), and differentiated human promyelocytic HL-60 leukemia cells (23) has been established

This work was supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society, by a National Cancer Institute of Canada Terry Fox Research Grant, and by the Canadian Foundation for AIDS Research. J.D.Y. is a Heritage Medical Scientist of the Alberta Heritage Foundation for Medical Research, and C.E.C. is a National Cancer Institute of Canada Terry Fox Cancer Research Scientist. S.Y.M.Y. was a recipient of a postgraduate studentship from the Croucher Foundation, Hong Kong.

ABBREVIATION: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

through direct measurements of [^{3}H]adenosine fluxes. In contrast, the conclusion that adenosine is an N2/*cit* and N4/*cit* permeant was based on results from indirect inhibition assays (4, 12, 13, 29) and not from direct measurements of [^{3}H]adenosine transport by N2(*cit*) and N4/*cit* processes. An ability to block transport does not necessarily establish that a substance is a permeant. For example, tubercidin inhibited Na^+-dependent thymidine transport in mouse intestinal cells but was not itself transported (4).

We used expression selection in *Xenopus laevis* oocytes to isolate a cDNA from rat jejunal epithelium encoding a pyrimidine-selective (N2/*cit*) Na^+-dependent nucleoside transporter termed rCNT1 (30).[1] The cDNA sequence of rCNT1 predicts a protein of 648 amino acids (relative molecular weight, 71,000) with 14 potential transmembrane domains. rCNT1, which is also expressed in kidney, is the first mammalian representative of a new transporter gene family that includes the *Escherichia coli* NUPC proton/nucleoside symporter (31). Recently, a cDNA encoding a second mammalian nucleoside transporter (SPNT) belonging to the rCNT1/NUPC family was isolated from rat liver (32). This protein, message for which was also detected in jejunum, mediates purine-selective N1/*cif* transport activity in oocytes and is predicted to have 662 amino acid residues, 64% of which are identical to those of rCNT1.

We describe an investigation of adenosine transport by recombinant rCNT1 expressed in *X. laevis* oocytes. Our results establish that adenosine binds to rCNT1 with high affinity and is a rCNT1 permeant but demonstrate that adenosine fluxes are 2 orders of magnitude lower than those for uridine. We also report the molecular cloning of SPNT (rCNT2 in our nomenclature) from rat jejunum and show that this nucleoside transporter mediates substantially greater fluxes of adenosine than are seen with rCNT1 under identical expression conditions.

Materials and Methods

rCNT1 and rCNT2 cDNAs. The isolation of plasmid pQQH1, which contains a 2.4-kb insert encoding rCNT1 in the expression vector pGEM-3Z (Promega, Madison, WI), by expression selection from a rat jejunal cDNA library was described previously (30). rCNT2 cDNA was obtained by reverse-transcription polymerase chain reaction amplification of total RNA from mucosal scrapings of Sprague-Dawley rat jejunum (25) using primers flanking the rat liver SPNT cDNA open reading frame (32). First-strand cDNA synthesis was performed on 5 μg of RNA as template using the SuperScript Preamplification System (GIBCO BRL, Baltimore, MD) and oligo(dT) as primer. The polymerase chain reaction (50 μl) contained 10 ng of template first-strand cDNA, 5 units of *Taq*/Deep Vent DNA polymerase (100:1), and 10 pmol each of primers corresponding to SPNT cDNA nucleotide positions 27–48 (sense, 5′-AAC CTC CAC TTC CTG CTT GTG A-3′) and nucleotide positions 2676–2694 (antisense, 5′-ACT TCT GTG AAA GAC TTC A-3′). Amplification for 1 cycle at 94° for 5 min, 55° for 1 min 20 sec, and 72° for 2 min and 29 cycles at 94° for 1 min, 55° for 1 min 20 sec, and 72° for 2 min produced a ~2.6-kb product that was ligated into the polymerase chain reaction vector pGEM-T (Promega) generating plasmid pAN1. The pAN1 insert was sequenced in both directions by *Taq* DyeDeoxy terminator cycle sequencing using an automated Model 373A DNA Sequencer (Applied Biosystems, Norwalk, CT).

[1] This was previously designated $cNT1_{rat}$.

Expression of recombinant proteins in *X. laevis* oocytes. Plasmids pQQH1 and pAN1 were linearized with *Xba*I and *Sal*I, respectively, and transcribed with T7 RNA polymerase in the presence of the m′GpppG cap (Ambion, Austin, TX) using the MEGAscript (Ambion) transcription system. Healthy stage VI oocytes of *X. laevis* treated with collagenase to remove follicular layers (25) were maintained at 18° in modified Barth's medium for 24 hr until injection with either 10 nl of rCNT1 or rCNT2 RNA transcript (1 ng/nl) or 10 nl of water. Injected oocytes were incubated for 3 days at 18° with a daily change of modified Barth's medium before the assay of transport activity.

Transport assays. Uptake of nucleosides by oocytes was traced with the respective ^{3}H-nucleoside (Moravek Biochemicals, Brea, CA) (5 μCi/ml), which were purified by high performance liquid chromatography before use. Assays were performed at 20° on groups of 10–12 oocytes in transport buffer (0.2 ml) containing either 100 mM NaCl or 100 mM choline chloride and 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.5 (30). At the end of the incubation, extracellular radioactivity was removed by six rapid washes in the appropriate ice-cold transport buffer. Individual oocytes were processed for quantification of oocyte-associated ^{3}H by liquid scintillation counting as described previously (30). In competition experiments, nonradioactive nucleosides (Sigma Chemical, St. Louis, MO) were added to oocytes at the same time as the ^{3}H permeant.

For efflux studies, groups of 20 rCNT1 RNA transcript-injected oocytes were preloaded with 10 μM [^{3}H]uridine (5 μCi/ml) in 0.2 ml of NaCl transport buffer at 20° for 30 min, followed by six rapid ice-cold washes in NaCl transport buffer to remove extracellular ^{3}H. One group of 20 oocytes was processed to determine the time-zero [^{3}H]uridine content (typically 50–60 pmol/oocyte) (30). The other groups of 20 oocytes were each resuspended in 1 ml of NaCl transport buffer (±1 mM nonradioactive nucleoside) at 20° to initiate efflux. At predetermined time intervals, duplicate 5-μl samples of incubation medium were removed and counted for ^{3}H. We established previously that uridine is only slowly metabolized by oocytes (25). In all experiments, oocytes were pretreated (30 min at 20°) with 1 μM deoxycoformycin to inhibit adenosine deaminase activity. This concentration of deoxycoformycin had no effect on rCNT1-mediated uridine fluxes (30).

Data analysis. Results for uptake experiments are given as mean ± standard error for 10–12 individual oocytes. Kinetic constants (apparent K_m and V_{max}), Na^+-activation parameters [K_{50} (Na^+ concentration at which the transport rate is half of its maximal value) and Hill coefficient], efflux rate constants (k) (mean ± standard error) were determined by nonlinear regression analysis (Enzfitter, Elsevier-Biosoft, Cambridge, UK). Data for efflux experiments are presented as plots of [^{3}H]uridine efflux (given as a percentage) versus time for groups of 20 oocytes. Each experiment was performed at least twice on different batches of oocytes.

Metabolism of adenosine in *X. laevis* oocytes. The metabolism of adenosine was measured in assay mixtures containing 25 oocytes, 10 μM [^{3}H]adenosine (Amersham Life Science, Clearbrook, IL; 23.0 Ci/mmol), and 1 μM deoxycoformycin in NaCl transport buffer (0.2 ml). After a 10-min incubation period at 20°, extracellular radioactivity was removed as described for transport assays, 100 μl of ice-cold 70% methanol solution was added, and the extraction mixtures were stored overnight at −20°. The extracts were recovered by centrifugation. Two equal portions were dried under a stream of nitrogen, and the residues were dissolved in 5 mM $NH_4H_2PO_4$, pH 4.0, or 10 mM KH_2PO_4 in 10% methanol. Nucleotides were analyzed by high performance liquid chromatography in one extract portion, using a Whatman Partisil 10 SAX anion exchange column (Whatman, Fairfield, NJ) with a Spectra-Physics high performance liquid chromatography system equipped with a P4000 pump, Rheodyne 7125 injection valve, UV 100 detector, and ChromJet integrator. The column was eluted (2 ml/min) with $NH_4H_2PO_4$ solutions, forming a linear gradient from 5 mM, pH 4.0, to 700 mM, pH 4.8, during a 30-min interval and with 700 mM $NH_4H_2PO_4$, pH 4.8, for an addi-

tional 10 min. The second extract portion was chromatographed on a Whatman Partisil 5 ODS-3 (reverse-phase) column that was eluted isocratically with a solution of 10 mM KH_2PO_4 in 10% methanol (1 ml/min) for the determination of nucleosides and nucleobases. Retention times of nucleotides, nucleosides, and nucleobases were determined by UV monitoring at 270 nm and comparison with reference standards. Fractions were collected at 20-sec intervals and mixed with 4 ml Ecolite (ICN Biomedicals Canada) for quantification of radioactivity by liquid scintillation methods. Radioactivity (cpm) in each fraction was converted to fmol after determination of the specific activity of the [^{3}H]adenosine permeant solution.

Results and Discussion

cDNAs have been isolated that encode transporter proteins corresponding to the two major Na^+-dependent nucleoside transporter subtypes implicated in adenosine transport in mammalian cells. We used expression selection in *X. laevis* oocytes to clone a cDNA encoding a pyrimidine-selective nucleoside transporter (rCNT1) from rat jejunum (30). The same cloning strategy was used subsequently to isolate a cDNA encoding a structurally related purine-selective nucleoside transporter (SPNT) from rat liver (32). rCNT1 is expressed in kidney as well as intestine (30). Northern analysis found message for SPNT in various tissues, including jejunum (32). We used reverse-transcription polymerase chain reaction amplification of RNA prepared from mucosal scrapings to confirm the presence of SPNT transcript in rat jejunum and to clone a cDNA for the full-length functional protein (rCNT2 in our nomenclature) from this tissue. The nucleotide sequence of the open reading frame of rCNT2 cDNA[2] was almost identical to that of SPNT, indicating that this transporter is expressed in both liver and jejunum of rats. The predicted amino acid sequence of rCNT2 was the same as that for SPNT except for conservative substitutions of glycine for alanine at residue 419 and valine for isoleucine at residue 522. These minor differences may reflect transporter genetic polymorphism (rCNT2 and SPNT cDNAs were cloned from Sprague-Dawley and Wistar rats, respectively). The presence of purine-selective and pyrimidine-selective nucleoside transporter processes in intestine have been demonstrated functionally in isolated mouse enterocytes (4) and rabbit brush-border membrane vesicles (7) and in oocytes injected with rat and rabbit intestinal mRNA (25–26).

Previous assignments of adenosine as a permeant of the pyrimidine-selective process in intestine and other mammalian cells and tissues were based on competition studies (i.e., the ability of adenosine to block transport activity in intact cells and membrane vesicles) and not on direct measurements of adenosine fluxes. The availability of rCNT1 and rCNT2 cDNAs from rat jejunum has given us a unique opportunity to study adenosine as a permeant of a recombinant pyrimidine-selective nucleoside transporter and to assess adenosine transportability by this protein relative to that by a recombinant purine-selective nucleoside transporter from the same tissue. We used a heterologous expression system, the *X. laevis* oocyte, that lacks detectable endogenous nucleoside transport activity (25, 30).

Fig. 1A shows a representative time course of [^{3}H]adenosine uptake in oocytes injected with either rCNT1 RNA transcript or water that demonstrates that recombinant rCNT1 mediated adenosine transport. Uptake of [^{3}H]adenosine (10 μM) was slower than that determined previously for uridine and thymidine under similar conditions (30) and after 10 min was 0.32 ± 0.04 pmol/oocyte, which was 6.3-fold higher than the control flux in water-injected oocytes (0.05 ±

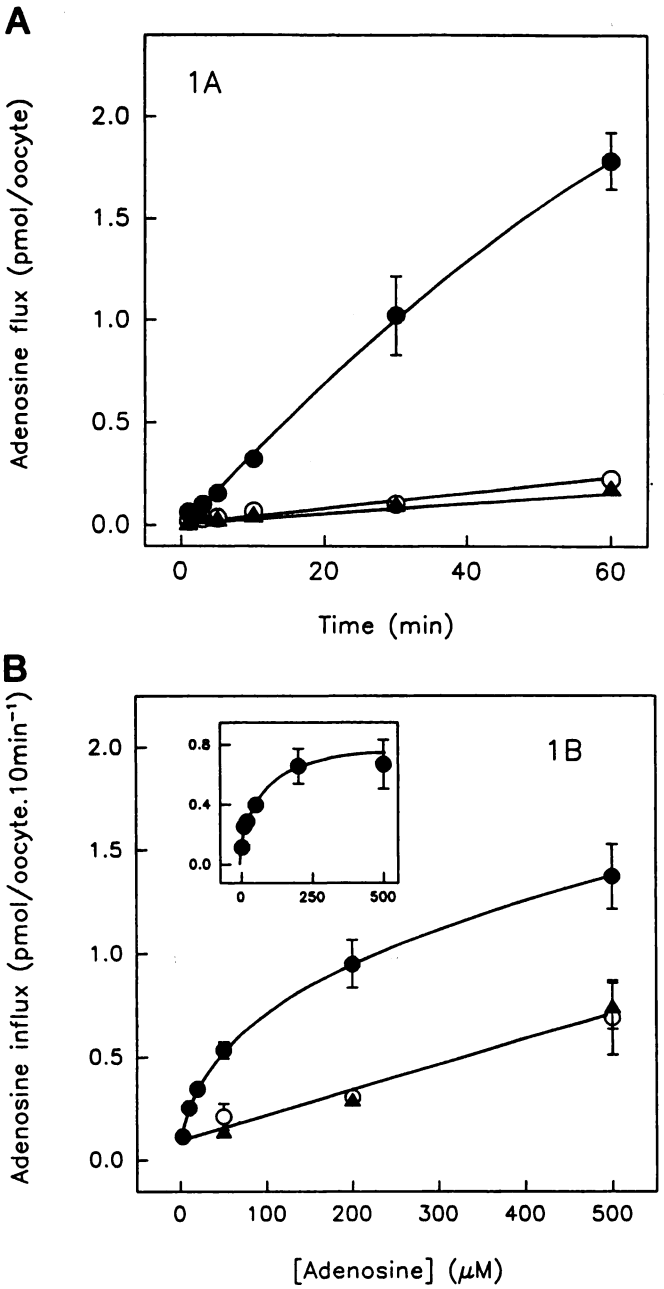

Fig. 1. Time course (A) and concentration dependence (B) of adenosine uptake by recombinant rCNT1 expressed in *X. laevis* oocytes. Oocytes injected with either 10 nl of water alone (▲) or 10 nl of water containing 10 ng of rCNT1 RNA transcript (●, ○) were incubated for 3 days at 18° in modified Barth's medium. Fluxes of adenosine (10 μM, 20°) for (A) 1–60 min or (B) 10 min were determined in transport buffer containing 100 mM NaCl (●, ▲) or 100 mM choline chloride (○). Each value is the mean ± standard error of 10–12 oocytes. B, *inset*, rCNT1-mediated fluxes of adenosine (influx in RNA transcript-injected oocytes in NaCl transport buffer minus influx in water-injected cells). Apparent K_m and V_{max} values (mean ± standard error), determined by nonlinear regression analysis (Enzfitter; Elsevier-Biosoft, Cambridge, UK), are presented in the text.

[2] The nucleotide sequence described in this article has been submitted to the GenBank/EMBL Data Bank (accession no. U66723.).

0.01 pmol/oocyte). Substitution of Na^+ in the incubation medium by choline$^+$ reduced the flux in RNA-injected oocytes by 80% to 0.07 ± 0.01 pmol/oocyte. Uptake of adenosine was linear during this time interval (10 min) and thus was used as the incubation period for subsequent initial rate measurements of adenosine influx. In seven independent experiments with different batches of oocytes, rCNT1-mediated fluxes of adenosine (10 μM), calculated as the difference in influx between RNA-injected and water-injected oocytes, ranged between 0.206 ± 0.098 and 0.509 ± 0.119 pmol/oocyte · 10 min^{-1} (~0.2-1.0 μM intracellular [^{3}H]adenosine).

Na^+-dependent, mediated influx of adenosine was saturable (Fig. 1B) and conformed to simple Michaelis-Menten kinetics with an apparent K_m value (26 ± 7 μM) that corresponded closely to that determined previously (30) for uridine (37 μM). The adenosine V_{max} value, however, was very much lower than the uridine V_{max} value (0.70 ± 0.05 pmol/oocyte · 10 min^{-1} or 0.070 ± 0.005 pmol/oocyte min^{-1} versus 21 pmol/oocyte min^{-1}) (30). Calculated V_{max}/K_m ratios were 0.003 pmol per oocyte $min^{-1}\cdot\mu M^{-1}$ (adenosine) and 0.57 pmol/oocyte $min^{-1}\cdot\mu M^{-1}$ (uridine), for a difference of 190-fold. To provide confirmatory evidence of high affinity binding of adenosine to rCNT1, graded concentrations (5 μM to 1 mM) of adenosine, thymidine, and unlabeled uridine were tested for their ability to block rCNT1-mediated influx of 10 μM [^{3}H]uridine (Fig. 2). Calculated apparent K_i values (assuming competitive inhibition and a uridine apparent K_m of 37 μM) were 29 ± 2, 23 ± 2, and 26 ± 2 μM, respectively, for adenosine, thymidine, and uridine. We also recently determined kinetic parameters for rCNT1-mediated transport of adenosine and uridine in transiently transfected COS-1 cells (33). Although the absolute V_{max} values in the two systems cannot be directly compared because of differences in cell size and amounts of recombinant protein, the K_m values, which represent a measure of transporter affinity, are directly comparable. The K_m values obtained for uridine in the two expression systems differed by only 2-fold, whereas those for adenosine were essentially the same, suggesting that recombinant rCNT1 functioned similarly in amphibian and mammalian cells. As was the case for *X. laevis* oocytes, the V_{max} value for adenosine transport in COS-1 cells was very much less than that determined for uridine in the same system (33). Permeants with similar K_m values but different V_{max} values have been observed with other transport systems (34, 35).

Influx of adenosine in water-injected oocytes was approximately linear over the concentration range studied, suggesting a lack of endogenous mediated transport of adenosine in oocytes (Fig. 1B). Adenosine is more lipophilic than uridine (36) and showed a greater nonsaturable uptake in water-injected oocytes. When measured in the same batch of cells at a concentration of 1 mM over an extended incubation time of 30 min, adenosine and uridine fluxes differed by 6-fold (18.8 ± 1.67 versus 3.06 ± 0.27 pmol/oocyte · 30 min^{-1}, respectively).

Classification of rCNT1 as a pyrimidine-selective nucleoside transporter was based on selective inhibition of uridine and thymidine fluxes by pyrimidine nucleosides and adenosine (adenosine, thymidine, uridine ≫ guanosine, inosine) (30). Consistent with these studies, rCNT1-mediated influx of 10 μM adenosine was strongly inhibited by 0.2 mM thymidine, cytidine, adenosine, and uridine (Fig. 3). In contrast, guanosine and inosine had no effect. Selectivity of the transporter for guanosine (a presumed N4/*cit* permeant) was also investigated by direct measurements of [^{3}H]guanosine (10 μM) fluxes in RNA transcript-injected and water-injected oocytes. A representative experiment gave fluxes of 0.045 ± 0.002 and 0.025 ± 0.002 pmol/oocyte · 10 min^{-1}, respectively. Mediated transport of guanosine (0.020 ± 0.008 pmol/oocyte · 10 min^{-1}) was therefore very much less than for

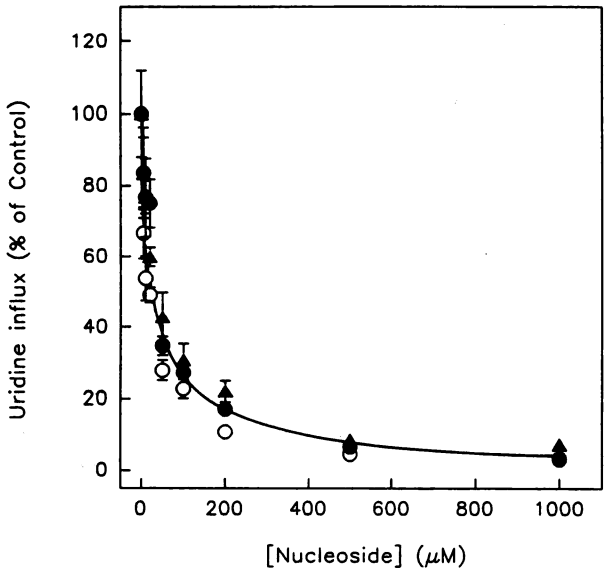

Fig. 2. Inhibition of rCNT1-mediated uridine influx by physiologic nucleosides. Uridine influx (10 μM at 20° for 1-min flux) was measured in rCNT1 RNA transcript-injected oocytes in the absence or presence of 10–1000 μM nonradioactive nucleosides (▲, adenosine; ○, thymidine; ●, uridine). Nonradioactive nucleosides were added to oocytes at the same time as [^{3}H]uridine. Fluxes were not corrected for the small (<1%) contribution of endogenous uridine uptake. Each value is the mean ± standard error of 10–12 oocytes. Apparent K_i values (mean ± standard error), calculated from IC_{50} values determined by nonlinear regression analysis (Enzfitter), are presented in the text.

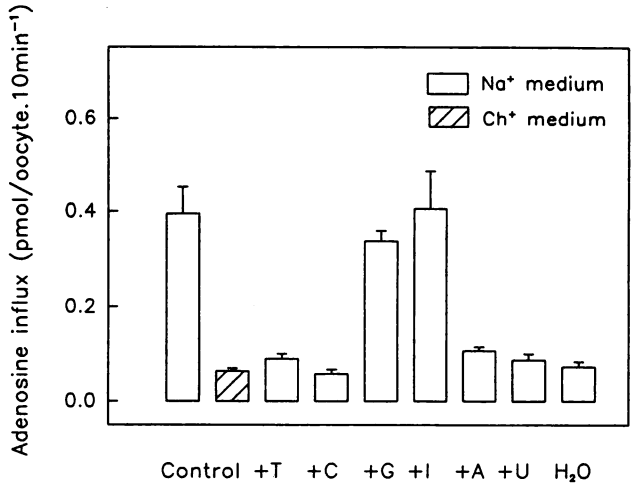

Fig. 3. Inhibition of rCNT1-mediated adenosine influx by physiological nucleosides. Adenosine influx (10 μM at 20° for 10-min flux) was measured in rCNT1 RNA transcript-injected oocytes in the absence (control) or presence of 0.2 mM of nonradioactive nucleosides (*T*, thymidine; *C*, cytidine; *G*, guanosine; *I*, inosine; *A*, adenosine; *U*, uridine). Nonradioactive nucleosides were added to oocytes at the same time as [^{3}H]adenosine. H_2O, water-injected oocytes. *Values*, mean ± standard error of 10–12 oocytes.

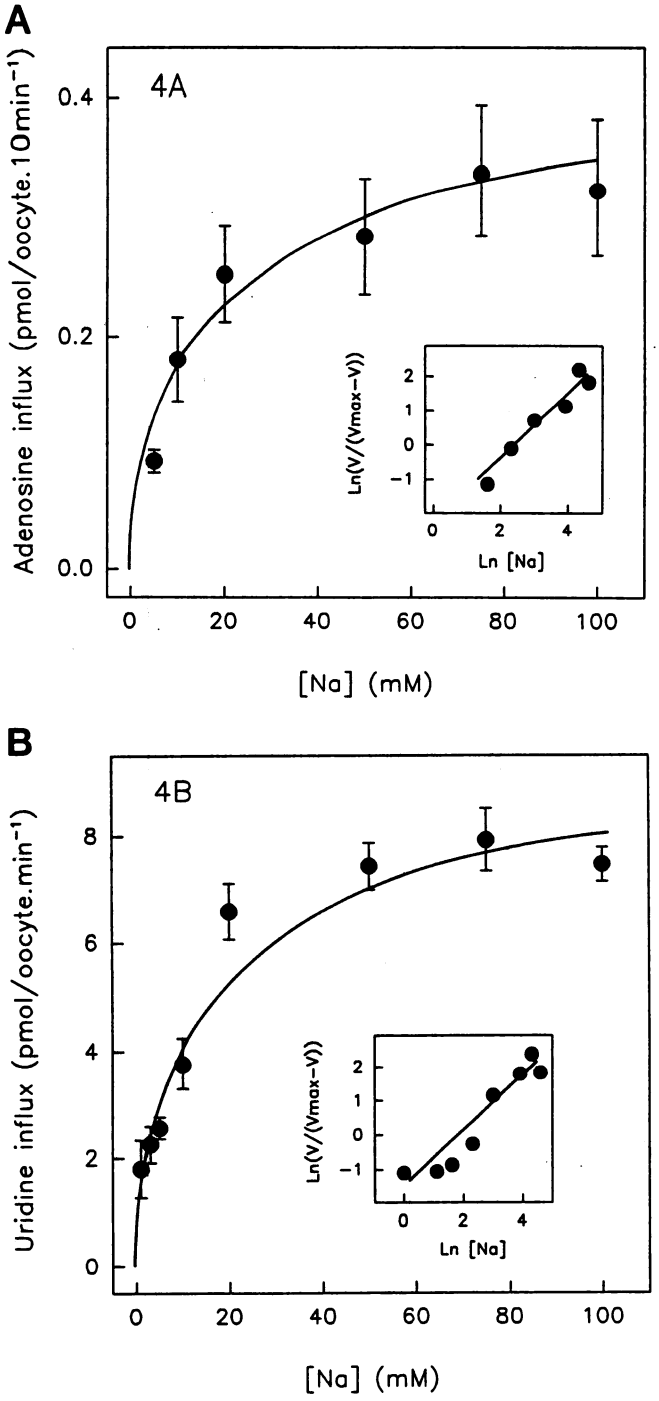

Fig. 4. Sodium dependence of rCNT1-mediated influx of (A) adenosine and (B) uridine. Adenosine and uridine influx (10 μM at 20° for 10- and 1-min fluxes, respectively) were measured in rCNT1 RNA transcript-injected oocytes in transport buffer containing 0–100 mM NaCl, using choline chloride to maintain isosmolality. Values for adenosine influx were corrected for endogenous adenosine uptake, which was not Na^+ dependent. A corresponding correction for uridine influx was not done because of the small (<1%) contribution of endogenous uridine uptake seen in water-injected oocytes. Each value is the mean ± standard error of 10–12 oocytes. *Insets*, Hill plots of the data. Hill coefficients and K_{50} values (mean ± standard error), determined by nonlinear regression analysis (Enzfitter), are presented in the text.

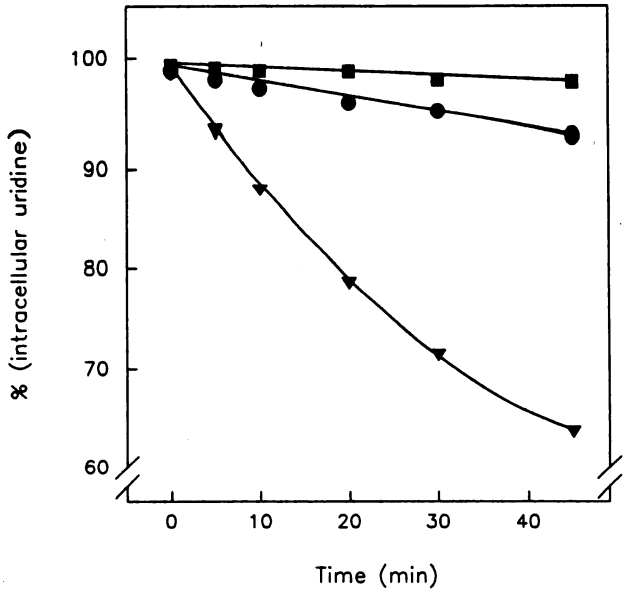

Fig. 5. The *trans*-stimulation and *trans*-inhibition of rCNT1-mediated uridine efflux by extracellular uridine and adenosine. Efflux of [^{3}H]uridine from rCNT1 RNA transcript-injected oocytes preloaded with 10 μM [^{3}H]uridine for 30 min at 20° was measured at 20° on groups of 20 oocytes suspended in (●) NaCl transport buffer alone or in NaCl transport buffer containing (▼) 1 mM uridine or (■) 1 mM adenosine.

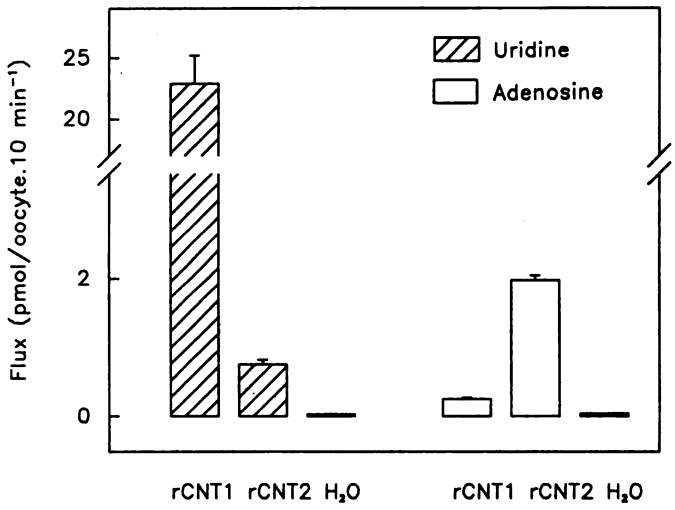

Fig. 6. Uptake of adenosine and uridine by recombinant rCNT1 and rCNT2 expressed in *X. laevis* oocytes. Oocytes injected with either 10 nl of water alone or 10 nl of water containing 10 ng of either rCNT1 or rCNT2 RNA transcript were incubated for 3 days at 18° in modified Barth's medium. Fluxes of adenosine and uridine (10 μM at 20° for 10 min) were determined in transport buffer containing 100 mM NaCl. *Values*, mean ± standard error of 10–12 oocytes.

adenosine. The predicted Na^+/permeant coupling stoichiometry of rCNT1, determined from Hill-type analysis of the relationship between nucleoside influx and Na^+ concentration, was 1:1 for both adenosine and uridine (Hill coefficients, 1.01 ± 0.12 and 1.12 ± 0.10, respectively; K_{50} values, 12.4 ± 2.6 and 9.5 ± 2.2 mM Na^+, respectively) (Fig. 4, A and B). Similar studies of uridine influx by the pyrimidine-selective nucleoside transport process in rat and bovine brush border membrane vesicles have also given Na^+/nucleoside coupling stoichiometries of 1:1 (12, 13) and there is evidence of the same coupling ratio for purine-selective system in different mammalian cells and tissues (13, 15–17, 20, 21).

For many transport processes, the V_{max} of zero-*trans* influx is thought to be determined by the rate of reorientation of the

unloaded transporter from the intracellular membrane face to the extracellular membrane face. Differences in V_{max} values among permeants, such as those found in the current study for adenosine versus uridine, could arise if the rate of conversion of the loaded transporter from its outward-facing conformation to its inward-facing conformation becomes rate limiting. The effects of adenosine and uridine on rCNT1 efflux were therefore investigated. Oocytes expressing recombinant rCNT1 were preloaded with 10 μM [^{3}H]uridine under conditions in which the majority of the accumulated intracellular radioactivity remained as unmetabolized uridine (25). Release of internalized [^{3}H]uridine from oocytes suspended in NaCl transport buffer was then measured in the presence and absence of extracellular nonradioactive adenosine and uridine to produce the efflux time courses shown in Fig. 5. Efflux of intracellular [^{3}H]uridine against the Na^+ electrochemical gradient was relatively slow (a 7.0% decrease in 45 min; efflux rate constant, k (10^{-3} min) = 1.31 ± 0.06). The addition of 1 mM uridine to the extracellular medium resulted in a large increase in uridine efflux (a 39% decrease in 45 min; k (10^{-3} min) = 10.2 ± 0.5). In marked contrast to the *trans*-stimulation seen with uridine, 1 mM extracellular adenosine (1 mM) caused a substantial *trans*-inhibition of uridine efflux (a 2.2% decrease in 45 min; k (10^{-3} min) = 0.35 ± 0.05). Thus, the rate of conversion of rCNT1 from its outward-facing conformation to its inward-facing conformation was increased when the transporter was complexed with uridine and decreased when it was complexed with adenosine.

Fig. 6 illustrates results of a direct comparison of rCNT1- and rCNT2-mediated transport of adenosine and uridine under identical conditions (10 μM, 10-min flux) in the same preparation of oocytes. rCNT1-mediated fluxes of adenosine and uridine were 0.214 ± 0.037 and 22.8 ± 0.5 pmol/oocyte · 10 min^{-1}, respectively, a difference of 2 orders of magnitude. rCNT2-mediated flux of adenosine was 1.94 ± 0.10 pmol/oocyte · 10 min^{-1}, demonstrating a large difference in expressed adenosine transport activity between the two recombinant NTs (rCNT1 < rCNT2). rCNT2-mediated flux of uridine was 0.75 ± 0.7 pmol/oocyte · 10 min^{-1}, demonstrating a large difference in uridine transport activity between the two nucleoside transporters but in the opposite direction (rCNT1 > rCNT2). The magnitude of rCNT2-mediated adenosine flux in Fig. 6 (1.9 pmol/oocyte · 10 min^{-1}) is in good agreement with the value (2.9 pmol/oocyte · 10 min^{-1}) calculated from the reported kinetic constants (32) for adenosine transport by recombinant rat liver SPNT (apparent K_m = 6 μM, V_{max} = 0.46 pmol/oocyte min^{-1}). The SPNT kinetic parameters (32) give a V_{max}/K_m ratio of 0.077 pmol/oocyte $min^{-1}\cdot\mu M^{-1}$, which is >20-fold higher than that determined here for adenosine transport by recombinant rCNT1 (0.003 pmol/oocyte $min^{-1}\cdot\mu M^{-1}$). The SPNT study (32) did not measure uridine fluxes, so our data present the first demonstration of uridine transport by this nucleoside transporter. Calculated uridine/adenosine flux ratios from Fig. 6 were 107 and 0.39 for rCNT1 and rCNT2, respectively. Therefore, although rCNT1 transported uridine very much faster than adenosine, rCNT2 transported adenosine only somewhat faster than uridine.

Fig. 7 shows the formation of adenine nucleotides during a 10-min incubation period with 10 μM [^{3}H]adenosine in oocytes expressing rCNT1 or rCNT2. Analysis of oocyte extracts on the anion exchange column indicated that the predominant metabolite in both assay mixtures was ATP, accompanied by traces of ADP and AMP. A small peak, with retention time at the position of dATP, appeared as a shoulder on the ATP peak. Analysis of the oocyte extracts on the reverse-phase column (not shown) revealed adenosine, inosine, and small amounts of hypoxanthine and adenine. These results suggest that adenosine is rapidly phosphorylated in *X. laevis* oocytes, and efficiently trapped as the 5′-triphosphate ester.

In conclusion, the current results provide a direct demonstration that mammalian Na^+-dependent pyrimidine-selective nucleoside transporters also transport adenosine. Uridine and adenosine were both transported by recombinant rCNT1 with high affinity and with the same apparent Na^+/nucleoside coupling ratio of 1:1. However, the apparent V_{max} value for adenosine transport was only 0.33% of that for uridine. At physiological extracellular concentrations of adenosine and uridine (high nanomolar to low micromolar values)(1, 2, 37–39), relative fluxes of the two nucleosides through the transporter will be determined by the ratio V_{max}/K_m, which differed by 2 orders of magnitude. Extracellular

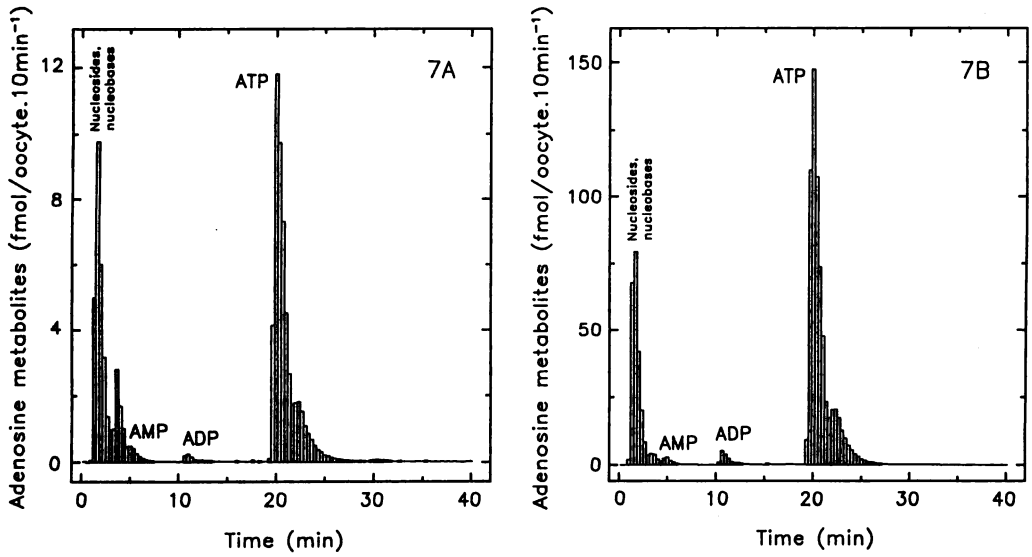

Fig. 7. Metabolism of adenosine in *X. laevis* oocytes expressing (A) rCNT1 or (B) rCNT2. Oocytes injected with either rCNT1 or rCNT2 RNA transcript were incubated with [^{3}H]adenosine (10 μM at 20° for 10 min) in transport buffer containing 100 mM NaCl and 1 μM deoxycoformycin. Methanolic extracts of the oocytes were analyzed by anion exchange high performance liquid chromatography. The chromatographic profiles are labeled with the positions of reference standards.

adenosine inhibited rCNT1-mediated uridine efflux from preloaded oocytes, which is consistent with a low rate of conversion of the rCNT1/adenosine complex from outward-facing to inward-facing conformations. Although rCNT1 and rCNT2 from rat jejunum were found to have overlapping selectivities for pyrimidine and purine nucleosides, the "purine-selective" transporter (rCNT2) had a greater tolerance for uridine as a permeant than did the "pyrimidine-selective" transporter (rCNT1) for adenosine. The relative contribution of rCNT1 to transmembrane Na^+-linked adenosine movements will depend on the extent to which rCNT2 (or other concentrative transporters that accept adenosine) are also expressed. Adenosine may, in some circumstances, function as an inhibitor of rCNT1 activity.

Acknowledgments

We thank Dawn Kieller for excellent technical assistance.

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