0022-3565/05/3122-601–608$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 312:601–608, 2005

Vol. 312, No. 2
73387/1193700
Printed in U.S.A.

Nucleoside Transport at the Blood-Testis Barrier Studied with Primary-Cultured Sertoli Cells

Ryo Kato, Tomoji Maeda, Toshihiro Akaike, and Ikumi Tamai

Department of Molecular Biopharmaceutics, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan (R.K., T.M., I.T.); and Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Kanagawa, Japan (R.K., T.A.)

Received July 5, 2004; accepted November 15, 2004

ABSTRACT

Nucleosides are essential for nucleotide synthesis in testicular spermatogenesis. In the present study, the mechanism of the supply of nucleosides to the testicular system across the blood-testis barrier was studied using primary-cultured Sertoli cells from rats and TM4 cells from mice. Uptake of uridine by these cells was time- and concentration-dependent. Uridine uptake was decreased under Na^+-free conditions, and the system was presumed to be high affinity, indicating an Na^+-dependent concentrative nucleoside transporter (CNT) is involved. On the other hand, nitrobenzylthioinosine, a potent inhibitor of Na^+-independent equilibrative nucleoside transporters (ENTs), inhibited uridine uptake by the Sertoli cells in a concentration-dependent manner. Expression of nucleoside transporters ENT1, ENT2, ENT3, CNT1, CNT2, and CNT3 was detected in Sertoli cells by reverse transcriptase-polymerase chain reaction analysis. Inhibition studies of the uptake of uridine by various nucleosides both in the presence and absence of Na^+ indicated that the most of those expressed nucleoside transporters, ENTs and CNTs, are involved functionally. These results demonstrated that Sertoli cells are equipped with multiple nucleoside transport systems, including ENT1, ENT2, and CNTs, to provide nucleosides for spermatogenesis.

Throughout the mammalian spermatogenic pathway, differentiating spermatogenic cells are located in close contact with somatic Sertoli cells, and their interaction has been considered to be essential for the proliferation, differentiation and survival of spermatogenic cells (Russel et al., 1990; Lui et al., 2003). Sertoli cells form tight junctions and act as a barrier to protect developing germ cells against harmful agents, while allowing the passage of nutrients, such as nucleosides, amino acids, glucose, and lactic acid, for spermatogenesis from circulating blood (Jutte et al., 1982; Bart et al., 2002). There are two routes for nucleotide synthesis, i.e., the de novo pathway and the salvage pathway. In the testis, it is thought that the salvage pathway is the more efficient route, and nucleotides are likely to be synthesized from incorporated nucleosides for the following reasons. The expression of amidophosphoribosyltransferase, an allosteric enzyme of de novo purine nucleotide biosynthesis, is low in rat testis (Iwahana et al., 1993). Rat testis has a high activity of hypoxanthine-guanine phosphoribosyltransferase and uridine kinase, rate-limiting enzymes of the purine and pyrimidine salvage pathways, respectively (Adams and Harkness, 1976; Haugen et al., 1988). Nucleoside incorporation into DNA or RNA in testis was observed by autoradiographic study (Monesi, 1962; Davies and Lawrence, 1981). Labeling with 5-bromodeoxyuridine, a pyrimidine analog, was found exclusively in the nuclei of rat Sertoli and spermatogenic cells after intraperitoneal injection of 5-bromodeoxyuridine (Shirataki et al., 1994). Accordingly, nucleosides must be supplied from circulating blood to inner cells across the blood-testis barrier (BTB).

Nucleosides are hydrophilic and require specific transporters for import through cell membranes. Mammalian nucleoside transporters have been characterized and identified at the molecular level (Griffith and Jarvis, 1996; Baldwin et al., 1999). These transporters are classified into the equilibrative and concentrative nucleoside transporter families. The equilibrative transport system is Na^+-independent and is inhibited by nitrobenzylthioinosine (NBMPR). This equilibrative transport system exhibits broad substrate selectivity and is divided into two types based on the sensitivity to NBMPR (Griffith and Jarvis, 1996; Cass et al., 1998), i.e., the

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by a grant from the Nakatomi Foundation.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.104.073387.

ABBREVIATIONS: BTB, blood-testis barrier; NBMPR, nitrobenzylthioinosine; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; FBS, fetal bovine serum; NMG, *N*-methyl-D-glucamine; DMSO, dimethylsulfoxide; RT, reversed transcription; PCR, polymerase chain reaction.

equilibrative and NBMPR-sensitive (*es*) system is inhibited by low concentrations of NBMPR, whereas the equilibrative and NBMPR-insensitive (*ei*) system is not. Two major equilibrative nucleoside transporters (ENTs), human ENT1 and ENT2, were identified as *es* and *ei* transporters, respectively (Griffiths et al., 1997a,b; Ward et al., 2000). Rat ENT1 and ENT2 also exhibited different sensitivity to NBMPR inhibition (Yao et al., 1997). The third member of the ENT family, ENT3, is not present at the cell surface but is located in some intracellular compartment (Hyde et al., 2001), and its functionality has not yet been characterized. There are also three Na^+-dependent concentrative nucleoside transporters (CNTs) (Wang et al., 1997; Gray et al., 2004). CNT1 and CNT2 preferentially transport pyrimidine nucleosides and purine nucleosides, respectively (Huang et al., 1994; Ritzel et al., 1998; Gerstin et al., 2002), whereas CNT3 transports both purine and pyrimidine nucleosides (Ritzel et al., 2001).

In the male reproductive organ, acquisition of sperm fertility proceeds during transit through the epididymal lumen. Adenosine is related to the acquisition of motility of sperm (Aitken et al., 1986; Vijayaraghavan and Hoskins, 1986), and nucleoside transport systems were characterized in rat epididymis epithelial cells (Leung et al., 2001). However, there is no information about nucleoside transport systems at the BTB, which is formed mainly by Sertoli cells. In the present study, to clarify the mechanism of the supply of nucleosides from the bloodstream to seminiferous tubule and spermatogenic cells, we investigated the transport of nucleosides in primary-cultured rat Sertoli cells and TM4 cells. The murine Sertoli-like cell line TM4 was derived from immature mouse testis and has a number of characteristics as Sertoli cells, including responsiveness to follicle-stimulating hormone and lack of response to luteinizing hormone, and enzyme and receptor expression patterns similar to primary Sertoli cell preparations (Mather et al., 1982). It was shown in the present study that primary-cultured rat Sertoli cells express ENT1, ENT2, ENT3, CNT1, CNT2, and CNT3, and kinetic analysis confirmed the involvement of plural transporters in uridine uptake by these cells.

Materials and Methods

Materials. [5-^{3}H]Uridine (16.2 Ci/mmol) and [carboxyl-^{14}C]inulin (2.5 mCi/g) were purchased from Moravek Biochemicals (Brea, CA) and American Radiolabeled Chemicals (St. Louis, MO), respectively. Uridine and NBMPR were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO), respectively. Collagenase and trypsin were obtained from Wako Pure Chemicals. All other reagents were purchased from Sigma-Aldrich and Wako Pure Chemicals.

Preparation and Primary Culture of Rat Sertoli Cells. Sertoli cells were isolated from 20-day-old Donryu rats (Saitama Experimental Animal Supply Co. Ltd., Saitama, Japan) according to the method reported by Nagao (1989) and Shiratsuchi et al. (1997). Briefly, testes were decapsulated, and seminiferous tubules were gently expressed and then incubated in 35 ml of 0.25% collagenase in phosphate-buffered saline for 20 min at 37°C with occasional stirring. The seminiferous tubules were washed with serum-free F12-L15 medium and then incubated with occasional gentle pipetting in 35 ml of 0.25% trypsin in phosphate-buffered saline for 20 min at 37°C. F12-L15 medium was composed of a 1:1 mixture of Ham's F12 medium (MP Biomedicals, Irvine, CA) and L-15 medium (MP Biomedicals), containing 15 mM HEPES, 10 unit/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). Trypsin treatment was terminated by adding 5 ml of FBS and 10 ml of F12-L15 medium containing 10% FBS. The resultant cell suspension was filtered twice through four sheets of gauze to remove cell aggregates and tissue debris, after which the cells were collected by centrifugation (1200 rpm × 10 min). The cells were suspended in 30 ml of F12-L15 medium containing 10% FBS and washed by centrifugation (900 rpm × 10 min). Finally, the cells were suspended in F12-L15 medium containing 10% FBS and passed once through nylon mesh (70 μm; BD Biosciences Discovery Labware, Bedford, MA). The isolated Sertoli cells thus obtained were cultured, and they adhered to the culture dish (353003; BD Biosciences Discovery Labware). Cells were grown in F12-L15 medium, containing 1 μg/ml norepinephrine, in a humidified incubator at 32.5°C for 3 days and at 37°C for 3 days. Sertoli cells in culture were isolated after removal with pipets of the spermatogenic cells floating on the surface of the coculture of testicular cells. About 90% of the cells adhering to the culture dish were Sertoli cells as judged from Nile red (Molecular Probes, Eugene, OR) staining, which is a marker for Sertoli cells (Mather et al., 1990). Six days after seeding, the cultures reached confluence and were used for the transport experiments and RT-PCR analysis. Spermatogenic cells were harvested at 4 days after seeding for RT-PCR. For the uptake experiment across the basolateral membrane of rat primary-cultured Sertoli cells, the cells were cultivated on 24-well Matrigel invasion chambers (BD Biosciences Discovery Labware) at 32.5°C for 3 days and at 37°C for 3 days.

TM4 Cell Culture. The murine Sertoli-like cell line TM4 cells, which were originally derived from mouse testis, were obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely grown in medium consisting of 50% Dulbecco's modified Eagle's medium (Invitrogen) and 50% F-12 medium containing 2.5% FBS, 5% horse serum (Invitrogen), 14 mM $NaHCO_3$, and 15 mM HEPES in a humidified incubator at 37°C under 5% CO_2.

Transport Experiments. For the transport experiments using primary-cultured rat Sertoli cells and TM4 cells, cells were harvested with cell scraper and suspended in transport medium containing 137 mM NaCl, 5 mM KCl, 0.39 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.95 mM $CaCl_2$, 0.8 mM $MgSO_4$, 25 mM D-glucose, and 10 mM HEPES, adjusted to pH 7.4. The cell suspension was preincubated at 37°C for 20 min in the transport medium, then centrifuged and the resultant cell pellets were resuspended in transport medium containing [^{3}H]uridine to initiate uptake. After a designated time, the cell suspension (200 μl) was diluted with 800 μl of ice-cold transport medium and centrifuged immediately (10,000 rpm × 1 min) to terminate the uptake reaction. Then, the cells were resuspended in ice-cold transport medium and obtained as the pellet after centrifugation. The resultant cell pellets were solubilized in 1 M NaOH, and the cell-associated radioactivity was measured by means of a liquid scintillation counter (Aloka, Tokyo, Japan) using Cleasol-1 (Nacalai Tesque, Kyoto, Japan) as a liquid scintillation fluid. Na^+-free transport medium was prepared by replacing 137 mM NaCl and 0.39 mM $NaHCO_3$ of the standard transport medium with 137 mM *N*-methyl-D-glucamine (NMG) and 0.39 mM $KHCO_3$, respectively, and was used to assess the uptake in the absence of sodium ion. NBMPR was dissolved in dimethylsulfoxide (DMSO) and was diluted to desired concentrations with transport medium, such that the final DMSO concentration was 0.5% or less. Time course of uridine uptakes were measured for 10 min. Na^+ dependence, concentration dependence, and inhibitory effect of NBMPR were measured at 2 or 5 min as described in the figures.

For the basolateral membrane uptake experiments, Matrigel invasion chambers (BD Biosciences Discovery Labware) were used. Matrigel invasion chamber is consisting of cell culture insert, which is coated with basement membrane matrix. Matrigel matrix is an extracellular matrix derived from Engelbreth-Holm-Swarm mouse and serves as a reconstituted basement membrane in vitro. Sertoli cells cultivated on Matrigel invasion chamber were used as polarized cells (Nakagawa et al., 2004). After preincubation in transport medium for 20 min, the medium in the basolateral membrane side was

replaced with transport medium containing [^{3}H]uridine. The apical-side medium was also replaced with transport medium. At 10 and 60 min, the cells on the invasion chamber were washed twice rapidly with ice-cold transport medium, solubilized in 1 M NaOH, and the cell-associated radioactivity was measured by means of a liquid scintillation counter. [^{3}H]Uridine uptake was corrected for extracellular binding based on the apparent uptake of [^{14}C]inulin, a membrane-impermeable marker.

RNA Isolation and RT-PCR. Total RNA was extracted from cultured cells with the ISOGEN RNA extraction solution (Wako Pure Chemicals) according to the manufacturer's protocol. cDNA was prepared from the extracted RNA by means of the reverse transcriptase reaction with SuperScript II RT (Invitrogen) and oligo(dT) primers according to the manufacturer's instructions. The cDNA was used for PCR amplification under the following conditions. Different sets of primers were designed and synthesized for PCR analysis of each transporter. The same primers were used for PCR reaction in rats and mice. Mouse CNT1 was not studied, since the complete cDNA sequence for mouse CNT1 has not been reported yet. The primer pair used for amplifying ENT1 was 5′-ACCATGATCAAGATTGTGCTCA and 5′-ATGAGATCCAACTTGGTCTCCT, which generated a 334-bp ENT1 PCR product. For ENT2, 5′-TTCGCTGCCCTTGCTATGCTCA and 5′-ACTGTGAAGACCAACACAA generated a 394-bp ENT2 PCR product. For ENT3, 5′-GTGGCTAACTTCCTGCTTGTCA and 5′-GGCCTCATGTAGTACCTGGCAT generated a 433-bp ENT3 PCR product. For CNT1, 5′-GGATGTCTTTGCCTTTCAGGT and 5′-CGATCCCAAAGGAGATGTAGG generated a 313-bp CNT1 PCR product. For CNT2, 5′-GGAAGAGTGACTTGTGCAA and 5′-GTGCTGGTATAGAGGTCACAGC generated a 300-bp CNT2 PCR product. For CNT3, 5′-CTGTCTTTTGGGGAATTGGA and 5′-CAGTAGTGGAGACTCTGTTT generated a 361-bp CNT3 PCR product. Reactions were carried out under the following conditions: denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min. Thirty cycles of reaction were performed. Primer pairs used for amplifying GAPDH were 5′-TGAAGGTCGGTGTGAACGGATTTGGC and 5′-CATGTAGGCCATGAGGTCCACCAC, which generated a 983-bp GAPDH PCR product, and the conditions were denaturation at 94°C for 30 s, annealing, and extension at 68°C for 2 min (total of 20 cycles). PCR products were analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide. The PCR products were sequenced and found to be correct.

Analytical Methods. Cellular protein content was determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard. Cellular uptake was usually expressed as cell/medium ratio (microliter per milligram of protein), which was obtained by dividing the uptake amount by the concentration of test compound in the transport medium. The apparent kinetic parameters K_m (Michaelis constant), V_{max} (maximal transport rate), and k_d (nonsaturable first order rate constant) of uridine uptake by Sertoli cells were calculated by nonlinear least square regression curve fitting according to the following Michaelis-Menten type equations, where v and $[s]$ are the velocity of substrate uptake and substrate concentration, respectively.

$$v = V_{max} \times [s]/(K_m + [s]) + k_d \times [s] \quad (1)$$

$$v = V_{max1} \times [s]/(K_{m1} + [s]) + V_{max2} \times [s]/(K_{m2} + [s]) + k_d \times [s] \quad (2)$$

$$v = V_{max1} \times [s]/(K_{m1} + [s]) + V_{max2} \times [s]/(K_{m2} + [s]) + V_{max3} \times [s]/(K_{m3} + [s]) + k_d \times [s] \quad (3)$$

When the data were fitted to eq. 2 or eq. 3 with two or three saturable transport components, the indices 1 to 3 indicate independent components, respectively. Nonlinear regression analysis was performed using the MULTI program (Yamaoka et al., 1981).

All data were expressed as means ± S.E.M., and statistical analysis was performed with Student's t test. The criterion of significance was taken to be $p < 0.05$.

Results

Time Course of Uridine Uptake in Sertoli Cells. Figure 1 shows the uptake of 0.07 μM [^{3}H]uridine by primary-cultured rat Sertoli cells (Fig. 1A) and TM4 cells (Fig. 1B) in the presence of Na^+ at 4°C or 37°C. Uptake of uridine by Sertoli cells at 37°C was significantly higher than that at 4°C. The uridine uptake by primary-cultured rat Sertoli cells and TM4 cells increased linearly up to 5 and 10 min, respectively. The same time course of the uptake of uridine was obtained at much higher substrate concentrations up to 200 μM, showing initial linear increases within 5 min and reaching steady state (data not shown). To avoid the influence of nonsaturable transport, initial uptake was analyzed at 2 or 5 min in the subsequent studies.

Concentration Dependence of Uridine Uptake in Sertoli Cells. To characterize the uridine transport in rat Sertoli cells and TM4 cells, the concentration dependence of uridine uptake was investigated. Uptake of uridine exhibited saturation in both rat Sertoli cells and TM4 cells (Fig. 2, A and C). In primary-cultured rat Sertoli cells, concentration dependence was measured in the presence and absence of Na^+ (Fig. 2, A and B). Under Na^+-free (NMG^+) condition, an Eadie-Hofstee plot for uptake by primary-cultured rat Sertoli cells, after correction for nonsaturable uptake (estimated from the first order rate constant obtained by nonlinear least-squares regression analysis), indicated an involvement of two saturable transport systems (Fig. 2B); the kinetic parameters were as follows: high-affinity transport system (K_{m1} = 14.5 ± 5.57 μM, V_{max1} = 160 ± 78.7 pmol/5 min/mg protein), low-affinity transport system (K_{m2} = 351 ± 609 μM, V_{max2} = 786 ± 1053 pmol/5 min/mg protein), and nonsaturable uptake (k_d = 0.60 ± 0.63 μl/5 min/mg protein). Fur-

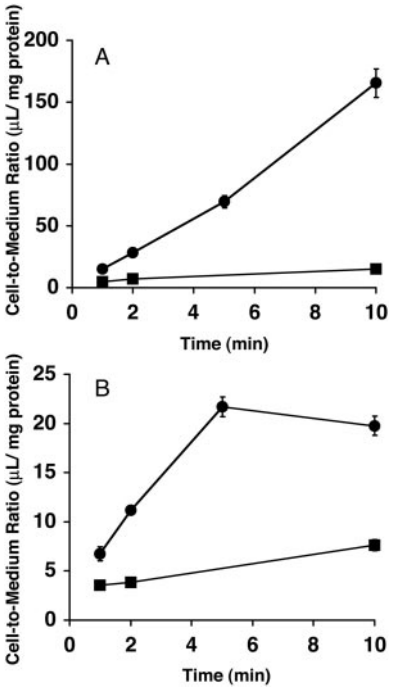

Fig. 1. Time course of [^{3}H]uridine uptake in Sertoli cells. Uptake of [^{3}H]uridine (0.07 μM) by primary-cultured rat Sertoli cells (A) and TM4 cells (B) was measured at 37°C (circles) or 4°C (squares). Cellular uptake is expressed as cell/medium ratio. Each result represents the means and S.E.M. (n = 3 or 4). If the S.E.M. is not shown, it is smaller than the symbols.

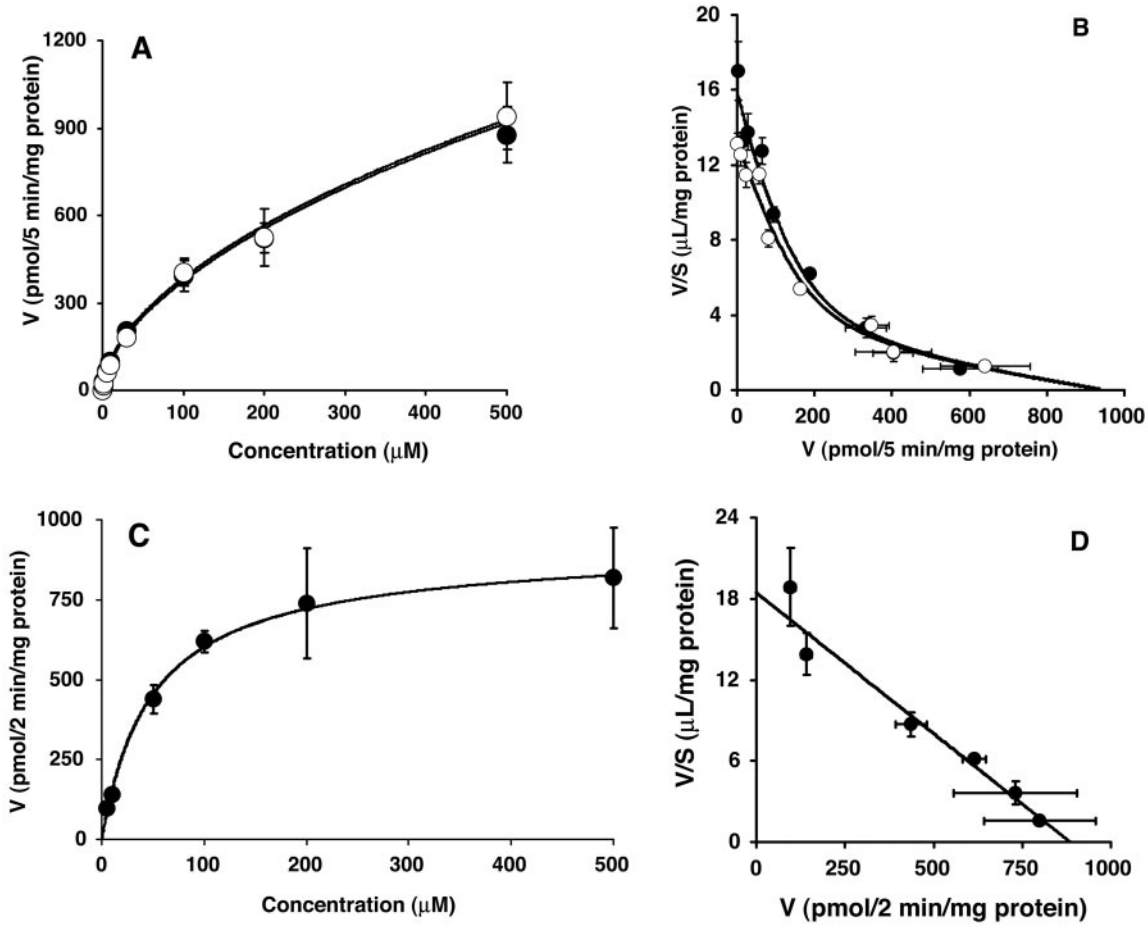

Fig. 2. Concentration dependence of uridine uptake in Sertoli cells. A and B, uptake of uridine by primary-cultured rat Sertoli cells in the presence (closed circle) or absence (open circle) of sodium ion at 37°C for 5 min. In the Na^+-free condition, Na^+ was replaced with NMG^+. C and D, uptake of uridine by TM4 cells was measured at 37°C for 2 min. B and D, Eadie-Hofstee plots of uridine uptake after correction for nonsaturable uptake evaluated from the first order rate constant obtained by nonlinear least-squares regression analysis as described under *Results*. Each result represents the means and S.E.M. (n = 3 or 4). If the S.E.M. is not shown, it is smaller than the symbols.

thermore, it was shown that Na^+-dependent transport component was involved in uridine transport by primary-cultured rat Sertoli cells (Fig. 2, A and B). After substitution of kinetic parameters (K_{m1}, V_{max1}, K_{m2}, V_{max2}, and k_d) of sodium-independent uridine transport obtained by eq. 2 to eq. 3, kinetic parameters for sodium-dependent transport system were calculated as follows, $K_{m3} = 4.17 \pm 4.13$ μM and $V_{max3} = 11.2 \pm 8.48$ pmol/5 min/mg protein.

In TM4 cells, uridine transport appeared to be mediated by one saturable component and one nonsaturable component (Fig. 2, C and D), and the kinetic parameters were obtained using eq. 1: $K_m = 48.0 \pm 10.3$ μM, $V_{max} = 885 \pm 149$ pmol/2 min/mg protein, and $k_d = 0.03 \pm 0.33$ μl/2 min/mg protein.

Characterization of Uridine Uptake in Sertoli Cells. To differentiate Na^+-dependent and -independent nucleoside transport systems, [^{3}H]uridine uptake was measured in the presence or absence of Na^+ in the transport medium. When extracellular Na^+ was replaced with NMG^+ at equimolar concentration, the uptake was reduced to 75% in primary-cultured rat Sertoli cells (Fig. 3A). From this result, at this uridine concentration, it was thought that 25 and 75% of apparent [^{3}H]uridine uptake are due to Na^+-dependent and -independent components, respectively, suggesting that both Na^+-dependent and -independent nucleoside transport systems are equipped in primary-cultured rat Sertoli cells. In

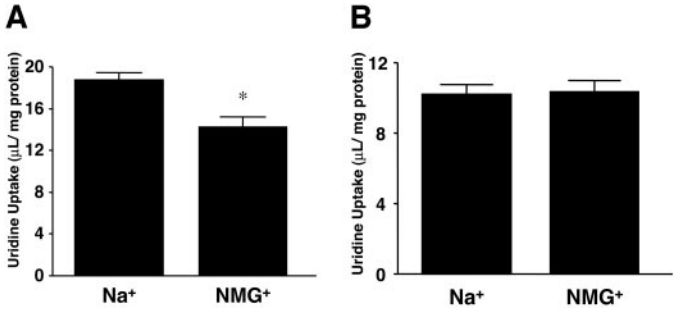

Fig. 3. Na^+ dependence of [^{3}H]uridine uptake in Sertoli cells. Uptake of [^{3}H]uridine (0.07 μM) was measured at 37°C for 2 min in primary-cultured rat Sertoli (A) or TM4 (B) cells, respectively. Cellular uptake is expressed as cell/medium ratio. In the Na^+-free condition, all Na^+ was replaced with NMG^+. Each column represents the mean and S.E.M. (n = 3 or 4). *, significant difference from the uptake in the presence of Na^+ (*, $p < 0.05$).

both the presence and absence of Na^+, [^{3}H]uridine uptake was inhibited by pyrimidine nucleosides, cytidine and thymidine, and the remaining uptake values were similar, suggesting that these nucleosides inhibited both Na^+-dependent and -independent uridine transport (Fig. 4). Since cytidine and thymidine were substrates for rat CNT1, it was suggested that rat CNT1 contributed to Na^+-dependent uridine uptake by primary-cultured rat Sertoli cells at lower uridine concen-

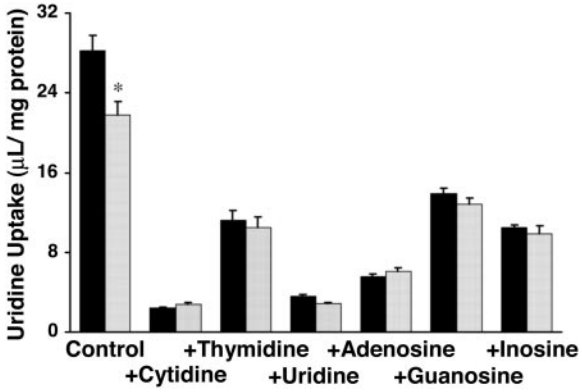

Fig. 4. Inhibitory effect of various nucleosides on [^{3}H]uridine uptake in primary-cultured rat Sertoli cells. Uptake of [^{3}H]uridine was measured for 5 min in primary-cultured rat Sertoli cells in the absence (dotted column) or presence (closed column) of Na^+. The concentrations of uridine were 0.07 μM. Cellular uptake is expressed as cell/medium ratio. Used concentration of guanosine and adenosine were 0.33 and 0.5 mM, respectively. Concentrations of other nucleosides were 1 mM. Each column represents the mean and S.E.M. (n = 3 or 4). *, significant differences from the uptake in the presence of Na^+ ($p < 0.05$).

tration. Similarly, purine nucleosides, adenosine, guanosine, and inosine, exhibited the same inhibitory effects, suggesting that rat CNT2 contributed to Na^+-dependent uridine uptake by primary-cultured rat Sertoli cells (Fig. 4). On the other hand, no significant decrease was observed in [^{3}H]uridine uptake by TM4 cells in the Na^+-free (NMG^+) condition, suggesting that there is negligible Na^+-dependent nucleoside transport system in TM4 cells (Fig. 3B).

Na^+-independent uptake was observed in both primary-cultured rat Sertoli cells and TM4 cells. To examine further whether the Na^+-independent uridine uptake is due to the ENTs, we employed NBMPR, a potent inhibitor of ENT. Na^+-independent nucleoside transport was pharmacologically defined by using different concentrations of NBMPR, since ENT1 (*es*) is inhibited by 100 nM NBMPR (Griffith and Jarvis, 1996; Cass et al., 1998). The remaining activity in the presence of 100 nM NBMPR was ascribed to ENT2 (*ei*) (Griffith and Jarvis, 1996; Cass et al., 1998). ENT2-mediated (*ei*) uridine transport in rats is resistant to 100 nM NBMPR but is inhibited by 0.5 mM NBMPR (Yao et al., 1997). In primary-cultured rat Sertoli cells, [^{3}H]uridine uptake was decreased significantly by 100 nM NBMPR, whereas 0.5 mM NBMPR exhibited greater inhibition in the absence of Na^+ at several concentration of uridine (5.0, 50.0, and 200.0 μM) (Fig. 5). For instance, at 5.0 μM uridine, uridine uptake was decreased in an NBMPR-concentration dependent manner to 36.3 and 13.4% of control at 100 nM and 0.5 mM, respectively. From kinetic parameters, at the same concentration of uridine, the relative contribution of high- and low-affinity transport components was estimated 67.1 and 18.0%, respectively. These results suggested that [^{3}H]uridine uptake is mediated by both ENT1 and ENT2 in primary-cultured rat Sertoli cells.

The Na^+-independent uptake of [^{3}H]uridine was suppressed by 100 nM and 0.5 mM NBMPR in TM4 cells to 34.4 and 6.0% of the control uptake, respectively (Fig. 6). Accordingly, it was suggested that the contributions of ENT1 and ENT2 to [^{3}H]uridine transport in TM4 cells amounted to 65.6% (i.e., 100 minus 34.4) and 28.4% (i.e., 34.4 minus 6.0), respectively, at this uridine concentration (0.07 μM).

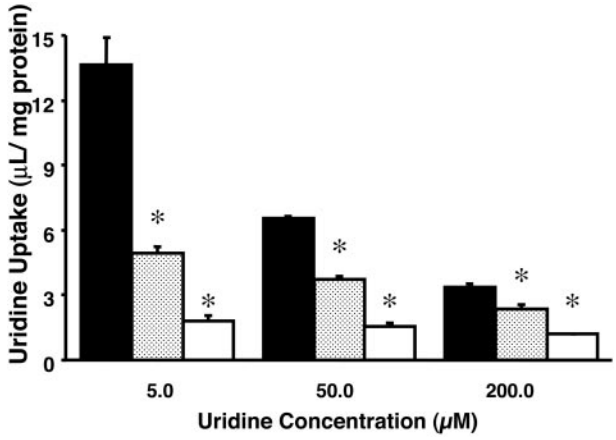

Fig. 5. Inhibitory effect of NBMPR on [^{3}H]uridine uptake in primary-cultured rat Sertoli cells. Uptake of [^{3}H]uridine was measured for 5 min in primary-cultured rat Sertoli cells in the absence or presence of NBMPR at 100 nM (dotted column) or 0.5 mM (open column). The concentrations of uridine were 5.0, 50.0, and 200.0 μM. Cellular uptake is expressed as cell/medium ratio. NBMPR was dissolved in DMSO and was diluted with transport medium (final DMSO concentration, 0.5%); 0.5% DMSO was included in the control experiment. Each column represents the mean and S.E.M. (n = 3 or 4). *, significant differences from the control uptake in the absence of Na^+ ($p < 0.05$).

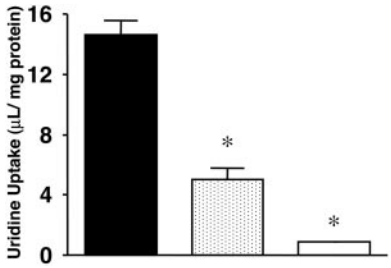

Fig. 6. Inhibitory effect of NBMPR on [^{3}H]uridine uptake in TM4 cells. Uptake of [^{3}H]uridine was measured for 5 min in TM4 cells in the absence or presence of NBMPR at 100 nM (dotted column) or 0.5 mM (open column). The concentrations of uridine were 0.07 μM. Cellular uptake is expressed as cell/medium ratio. NBMPR was dissolved in DMSO and was diluted with transport medium (final DMSO concentration, 0.5%); 0.5% DMSO was included in the control experiment. Na^+ was replaced with NMG^+. Each column represents the mean and S.E.M. (n = 3 or 4). *, significant differences from the control uptake in the absence of Na^+ ($p < 0.05$).

Basolateral Membrane Uptake of Uridine in Primary-Cultured Rat Sertoli Cells. Sertoli cells form tight junctions and are polarized, in that they show different characteristics at the basolateral and apical membranes, which face the blood side and the seminiferous tubular lumen, respectively. Thus, it is important to examine the polarity of nucleoside transport. To evaluate uptake of nucleosides from the blood side, uptake of [^{3}H]uridine from the basolateral membrane side of primary-cultured rat Sertoli cells was measured by cultivation of the cells on a Matrigel invasion chamber. Permeability of [^{14}C]inulin (0.11 ± 0.01 μl/cm^2/min) and transcellular electric resistance (196.5 ± 5.3 ohm $\cdot$ cm^2) in the presence of Sertoli cells were well corresponded to the reported values, including permeability of [^{3}H]inulin (<8.4%) and electric resistance (>about 52.2 ohm $\cdot$ cm^2) by Onoda et al. (1990), cited in the reference, who established this culture system, suggesting that tight junction is formed in the present study. It was also reported that Sertoli cells cultivated on a Matrigel invasion chamber are polarized (Nakagawa et al., 2004). Accordingly, it was considered that Sertoli

cells are polarized on Matrigel invasion chamber in this study. [^{3}H]Uridine uptake was significantly inhibited by unlabeled uridine at both 10 and 60 min (Fig. 7, A and B). Uptake of [^{3}H]uridine was decreased to 15.2% of control by 0.5 mM NBMPR, indicating that an equilibrative transport system is involved in basolateral membrane uptake of uridine (Fig. 7B).

Identification of Nucleoside Transporters in Sertoli and Germ Cells. Because the nucleoside transport activity in primary-cultured rat Sertoli cells exhibited characteristics consistent with those of CNTs and ENTs, the expression levels of ENT1, ENT2, ENT3, CNT1, CNT2, and CNT3 were examined by RT-PCR. As shown in Fig. 8, expression of all ENT and CNT family transporters was detected in primary-cultured rat Sertoli cells (lane 2). In TM4 cells, expression of ENT1, ENT2, ENT3, and CNT2 was observed (lane 5). In addition, all ENTs and CNTs were expressed in primary-cultured rat germ cells (lane 3). Since the complete cDNA sequence of mouse CNT1 has not been cloned, its expression was not examined (lanes 4 and 5).

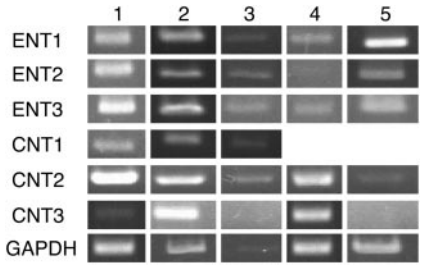

Fig. 8. Expression of nucleoside transporters in Sertoli cells and germ cells. Detection of CNT and ENT family transporters was carried out by RT-PCR using cDNA obtained from 6-day-cultured primary rat Sertoli cells and TM4 cells and 4-day-cultured primary rat germ cells. Positive controls with rat and mouse intestinal cDNA indicate the size of the amplified products. Primer sequences and amplification conditions were as described under *Materials and Methods*. PCR products were analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide. Lane 1, rat small intestine; lane 2, primary-cultured rat Sertoli cells; lane 3, primary-cultured rat germ cells; lane 4, mouse small intestine; lane 5, TM4 cells.

Discussion

In the present study, we focused on testicular transport of nucleosides because large amounts of nucleosides are required for proliferation and differentiation of spermatogenic cells and for Sertoli cells. The BTB is mainly formed by Sertoli cells and limits transfer of compounds between the bloodstream and seminiferous tubular lumen. To understand the transport mechanism of nucleosides at the BTB, it is important to measure the uptake of nucleosides from the bloodstream into Sertoli cells. Primary-cultured rat Sertoli cells and TM4 cells derived from mouse Sertoli cells were used as models of the BTB. Uridine was also used as a typical substrate of nucleoside transporters (Griffith and Jarvis, 1996; Baldwin et al., 1999).

Uptake of uridine by rat Sertoli cells in primary culture exhibited significant temperature dependence and was saturable, demonstrating an involvement of a carrier-mediated transport mechanism (Fig. 1A). Kinetic analysis suggested that uridine uptake was mediated by three different transport systems, one sodium-dependent high-affinity transport system and two sodium-independent transport systems (Fig. 2, A and B), whereas the analysis may not be reliable due to an involvement of many kinetic parameters to be estimated. Uptake of uridine by TM4 cells was also temperature-dependent, but in contrast to the rat Sertoli cells in primary culture, a single saturable component was observed with a K_m of 48 μM for uridine (Figs. 1B and 2, C and D). Gene expression of rat CNT1, CNT2, CNT3, ENT1, ENT2, and ENT3 was obtained by RT-PCR analysis in primary-cultured rat Sertoli cells (Fig. 8). These results are consistent with the functional studies such as sodium dependence, inhibition assay, and kinetic analysis. It has been reported that rat CNTs have high affinity for uridine (K_m = 31 and 37 μM) (Huang et al., 1994; Ritzel et al., 1998), whereas rat ENTs have lower affinity (K_m = 150 and 300 μM) (Yao et al., 1997). Kinetic parameters estimated in the present study (K_{m1} = 14.5 μM and K_{m2} = 351 μM (sodium-independent) and K_{m3} = 4.17 μM (sodium-dependent) are similar to those of ENT1, ENT2, and CNT-type transporters, respectively. From the inhibitory effects of various nucleosides on uridine uptake, it was suggested that at least CNT1 and CNT2 were involved in Na^+-dependent uridine uptake by primary-cultured rat Sertoli cells (Fig. 4). Since human and mouse CNT3 transports these nucleosides, including guanosine, inosine, cytidine, and thymidine, it was speculated that rat CNT3 also transports these nucleosides. However, at present, there is no information on the functionality of rat CNT3. Accordingly, although it is not conclusive at present, it was thought that rat CNT3 may contribute to sodium-dependent uridine uptake considering from the inhibitor effects. The apparent discrepancy in the extent of contribution of sodium-dependent transport between kinetic analysis (16%) and inhibition study (25%) may be partly due to that sodium-dependent uptake was apparently evaluated by a single component, although inhibition assay and RT-PCR analysis indicated involvement of two or three CNTs. In the absence of Na^+, uptake of uridine was maintained about 75% of that in the presence of sodium ion (Fig. 3A) and broadly inhibited by various nucleosides (Fig. 4). The effect of NBMPR was concentration-dependent, suggesting that both NBMPR-sensitive (*es*), and NBMPR-insensitive equilibrative (*ei*) nucleoside transporters contribute to uridine uptake by rat Sertoli cells (Fig. 5). Since rat

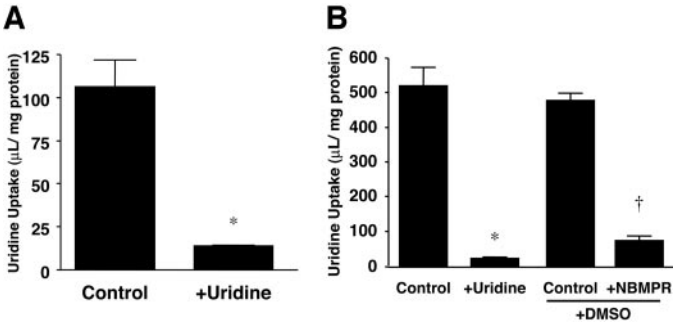

Fig. 7. Inhibitory effects of uridine or NBMPR on the basolateral membrane uptake of [^{3}H]uridine by primary-cultured rat Sertoli cells. Uptake of [^{3}H]uridine (0.07 μM) by Sertoli cells cultivated on Matrigel invasion chambers was measured at 37°C for 10 (A) or 60 min (B). Cellular uptake is expressed as cell/medium ratio. When the effect of NBMPR was examined, 0.5% DMSO was used in the control (+DMSO); see the legend to Fig. 5. [^{3}H]Uridine uptake was corrected for extracellular adhesion based on the apparent uptake of [^{14}C]inulin, a membrane-impermeable marker. Each column represents the mean and S.E.M. (n = 3 or 4). * and †, significant differences from the control or control (+DMSO) condition, respectively ($p < 0.05$).

ENT1 and rat ENT2 have different sensitivity to NBMPR, with IC_{50} values of 4.6 nM and >1 μM, respectively (Yao et al., 1997), the Na^+-independent uridine uptake by rat Sertoli cells was mediated by both ENT1 and ENT2. From kinetic parameters, estimated contribution of high- and low-affinity transport components, ENT1 and ENT2, was corresponded to the results of inhibitory effect of NBMPR. Since ENT3 might be localized intracellularly (Hyde et al., 2001), it may not be involved in cellular uptake of uridine. Our results showed that the sodium-independent transport, presumably due to ENT1 and ENT2, was predominantly contributed to uridine uptake in rat Sertoli cells.

In contrast, sodium-dependent uridine transport activity was not observed in TM4 cells (Fig. 3B), and expression of CNTs was very weak or undetectable (Fig. 8). These results indicated that uridine uptake is mainly mediated by equilibrative transporters. Kinetics analysis showed that uridine uptake is mediated by single component (Fig. 2, C and D), whereas RT-PCR analysis showed gene expression of mouse ENT1 and ENT2 in TM4 cells (Fig. 8). One possible reason for this apparent discrepancy may be due to the similarity of K_m values between mouse ENT1 and ENT2, but we cannot discuss this point since substrate affinities of ENT1 and ENT2 have not been reported yet. However, mouse ENT1 and ENT2 have different sensitivity to NBMPR, with IC_{50} values of 1.4 nM and >10 μM, respectively (Kiss et al., 2000), and the inhibitory effects of different concentration of NBMPR were examined in the present study. Since the observed effect of NBMPR was concentration-dependent, it was suggested that equilibrative transporters, ENT1 and ENT2, contribute to uridine uptake in TM4 cells (Fig. 6). It is not clear whether the differences of transport activities and expression profiles of nucleoside transporters between primary-cultured rat Sertoli cells and TM4 cells are due to species difference or differences in the age of animals and/or cell culture conditions. In the rat liver, the expression pattern of nucleoside transporters is developmentally regulated (del Santo et al., 2001). Adult rat hepatocytes show Na^+-dependent uridine uptake, whereas fetal hepatocytes exhibit NBMPR-sensitive equilibrative Na^+-independent transport. Therefore, it is thought that the expression pattern of nucleoside transporters is also affected by cell development and aging.

It is thought that the nucleoside transport activity observed in Sertoli cells corresponds to the flux from the bloodstream to testicular tissues for DNA and RNA synthesis. In the present study, Matrigel invasion chambers, coated with extracellular matrix, were used for measurement of the directional uptake of uridine. In the testis, the basal lamina is composed of basement membrane, which is comprised of type IV collagen, laminin, heparan sulfate proteoglycan (Hadley and Dym, 1987), and entactin (Lian et al., 1992); a thin collagen layer; and a layer of peritubular myoid cells (Dym, 1994). The Matrigel matrix serves as a reconstituted basement membrane derived from Engelbreth-Holm-Swarm mouse sarcoma, containing laminin, type IV collagen, entactin, and heparan sulfate proteoglycan (Kleinman et al., 1982). Primary-cultured rat Sertoli cells cultivated on Matrigel invasion chambers have been used as polarized cell monolayers (Onoda et al., 1990; Okanlawon and Dym, 1996; Nakagawa et al., 2004). As shown in Fig. 7, basolateral membrane uptake of [^{3}H]uridine was observed, and this uptake was inhibited by an excess of unlabeled uridine and by NBMPR. These results suggested that at least ENT1 and/or ENT2 are involved in uridine uptake from the basolateral side in primary-cultured rat Sertoli cells.

Figure 8 showed that all CNT and ENT genes were expressed in primary-cultured rat spermatogenic cells as well as Sertoli cells. Time- and temperature-dependent uridine uptake was observed, and an excess of unlabeled uridine and NBMPR inhibited this uptake (data not shown), suggesting that the nucleoside transporters take up essential nucleosides for spermatogenesis. In primary-cultured rat epididymal epithelial cells, ENT1, ENT2, and CNT2 are expressed and sodium-dependent transporter CNT2 mainly mediates uridine uptake (Leung et al., 2001). Thus, Sertoli cells and epididymal cells appear to have in common CNT2, but the contribution of other nucleoside transporters may be different between these two types of cells, although both of these cells should contribute differentiation and maturation of spermatogenic cells.

In summary, we have shown that primary-cultured rat Sertoli cells express ENT1, ENT2, ENT3, CNT1, CNT2, and CNT3 and exhibit nucleoside uptake activities mediated by ENT1, ENT2, and CNT-type transporters. The uridine uptake system involves kinetically three components, and their characteristics and kinetic parameters corresponded to those of CNTs and ENTs. It was also shown that uridine is taken up from basolateral side into Sertoli cells via ENTs. Since one of the roles of Sertoli cells is to provide nutrients and metabolic precursors to spermatogenic cells located within the BTB, nucleosides taken up by nucleoside transporters are presumably used for DNA and RNA synthesis in Sertoli cells and spermatogenic cells. The mechanism that mediates the efflux of nucleosides from Sertoli cells to the seminiferous tubular lumen for spermatogenetic cells remains to be established.

Acknowledgments

We thank Yoshinobu Nakanishi and Akiko Shiratsuchi (Kanazawa University) for helpful advice on primary culture of rat Sertoli cells.

References

Adams A and Harkness RA (1976) Developmental changes in purine phosphoribosyltransferases in human and rat tissues. *Biochem J* **160:**565–576.

Aitken RJ, Mattei A, and Irvine S (1986) Paradoxical stimulation of human sperm motility by 2-deoxyadenosine. *J Reprod Fertil* **78:**515–527.

Baldwin SA, Mackey JR, Cass CE, and Young JD (1999) Nucleoside transporters: molecular biology and implications for therapeutic development. *Mol Med Today* **5:**216–224.

Bart J, Groen HJ, van der Graaf WT, Hollema H, Hendrikse NH, Vaalburg W, Sleijfer DT, and de Vries EG (2002) An oncological view on the blood-testis barrier. *Lancet Oncol* **3:**357–363.

Cass CE, Young JD, and Baldwin SA (1998) Recent advances in the molecular biology of nucleoside transporters of mammalian cells. *Biochem Cell Biol* **76:**761–770.

Davies AG and Lawrence NR (1981) Timing and localization of the stimulatory effect of follicle-stimulating hormone on uridine incorporation in the mouse testis in vivo. *J Endocrinol* **88:**443–449.

del Santo B, Tarafa G, Felipe A, Casado FJ, and Pastor-Anglada M (2001) Developmental regulation of the concentrative nucleoside transporters CNT1 and CNT2 in rat liver. *J Hepatol* **34:**873–880.

Dym M (1994) Basement membrane regulation of Sertoli cells. *Endocr Rev* **15:**102–115.

Gerstin KM, Dresser MJ, and Giacomini KM (2002) Specificity of human and rat orthologs of the concentrative nucleoside transporter, SPNT. *Am J Physiol Renal Physiol* **183:**F344–F349.

Gray JH, Owen RP, and Giacomini KM (2004) The concentrative nucleoside transporter family, SLC28. *Eur J Physiol* **447:**728–734.

Griffith DA and Jarvis SM (1996) Nucleoside and nucleobase transport systems of mammalian cells. *Biochim Biophys Acta* **1286:**153–181.

Griffiths M, Beaumont N, Yao SY, Sundaram M, Boumah CE, Davies A, Kwong FY, Coe I, Cass CE, Young JD, et al. (1997a) Cloning of a human nucleoside trans-

porter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nat Med* **3:**89–93.
Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, and Baldwin SA (1997b) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. *Biochem J* **328:**739–743.
Hadley MA and Dym M (1987) Immunocytochemistry of extracellular matrix in the lamina propria of the rat testis: electron microscopic localization. *Biol Reprod* **37:**1283–1289.
Haugen TB, Hansson V, and Fritzson P (1988) Cellular distribution, developmental changes and effects of cryptorchidism on uridine kinase in rat testis. *J Reprod Fert* **83:**655–661.
Huang QQ, Yao SY, Ritzel MW, Paterson AR, Cass CE, and Young JD (1994) Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein. *J Biol Chem* **269:**17757–17760.
Hyde RJ, Cass CE, Young JD, and Baldwin SA (2001) The ENT family of eukaryote nucleoside and nucleobase transporters. *Mol Membr Biol* **18:**53–63.
Iwahana H, Yamaoka T, Mizutani M, Mizusawa N, Ii S, Yoshimoto K, and Itakura M (1993) Molecular cloning of rat amidophosphoribosyltransferase. *J Biol Chem* **268:**7225–7237.
Jutte NH, Jansen R, Grootegoed JA, Rommerts FF, Clausen OP, and van der Molen HJ (1982) Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. *J Reprod Fert* **65:**431–438.
Kiss A, Farah K, Kim J, Garriock RJ, Drysdale TA, and Hammond JR (2000) Molecular cloning and functional characterization of inhibitor-sensitive (mENT1) and inhibitor-resistant (mENT2) equilibrative nucleoside transporters from mouse brain. *Biochem J* **352:**363–372.
Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, and Martin GR (1982) Isolation and characterization of type IV procollagen, laminin and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry* **21:**6188–6193.
Leung GP, Ward JL, Wong PY, and Tse CM (2001) Characterization of nucleoside transport systems in cultured rat epididymal epithelium. *Am J Physiol Cell Physiol* **280:**C1076–C1082.
Lian G, Miller KA, and Enders GC (1992) Localization and synthesis of entactin in seminiferous tubules of the mouse. *Biol Reprod* **47:**316–325.
Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with folin phenol reagent. *J Biol Chem* **193:**265–267.
Lui WY, Mruk D, Lee WM, and Cheng CY (2003) Sertoli cell tight junction dynamics: their regulation during spermatogenesis. *Biol Reprod* **68:**1087–1097.
Mather JP, Attie KM, Woodruff TK, Rice GC, and Phillips DM (1990) Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. *Endocrinology* **127:**3206–3214.
Mather JP, Zhuang LZ, Perez-Infante V, and Phillips DM (1982) Culture of testicular cells in hormone-supplemented serum-free medium. *Ann NY Acad Sci* **383:**44–68.
Monesi V (1962) Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. *J Cell Biol* **14:**1–18.
Nagao Y (1989) Viability of meiotic prophase spermatocytes of rats is facilitated in primary culture of dispersed testicular cells on collagen gel by supplementing epinephrine or norepinephrine: evidence that meiotic prophase spermatocytes complete meiotic divisions in vitro. *In Vitro Cell Dev Biol* **25:**1088–1098.
Nakagawa A, Nagaosa K, Hirose T, Tsuda K, Hasegawa K, Shiratsuchi A, and Nakanishi Y (2004) Expression and function of class B scavenger receptor type I on both apical and basolateral sides of the plasma membrane of polarized testicular Sertoli cells of the rat. *Dev Growth Differ* **46:**283–298.
Okanlawon A and Dym M (1996) Effect of chloroquine on the formation of tight junctions in cultured immature rat Sertoli cells. *J Androl* **17:**249–255.
Onoda M, Suarez-Quian CA, Djakiew D, and Dym M (1990) Characterization of Sertoli cells cultured in the bicameral chamber system: relationship between formation of permeability barriers and polarized secretion of transferrin. *Biol Reprod* **43:**672–683.
Ritzel MW, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, Ritzel RG, Mowles DA, Carpenter P, Chen XZ, et al. (2001) Molecular identification and characterization of novel human and mouse concentrative Na^+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (System cib). *J Biol Chem* **276:**2914–2927.
Ritzel MW, Yao SY, Ng AM, Mackey JR, Cass CE, and Young JD (1998) Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na^+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. *Mol Membr Biol* **15:**203–211.
Russel LD, Ettlin RA, Hikim AP, and Clegg ED (1990) The Sertoli cell, in *Histological and Histopathological Evaluation of the Testis*, pp 29–36, Cache River Press, Clearwater, FL.
Shirataki S, Takihara H, Imoto K, Kamada K, Baba Y, and Naito K (1994) Quantitative analysis of spermatogenic DNA synthesis in the rat using a monoclonal anti-5-bromodeoxyuridine antibody. *Andrologia* **26:**343–349.
Shiratsuchi A, Umeda M, Ohba Y, and Nakanishi Y (1997) Recognition of phosphatidylserine on the surface of apoptotic spermatogenic cells and subsequent phagocytosis by Sertoli cells of the rat. *J Biol Chem* **272:**2354–2358.
Vijayaraghavan S and Hoskins DD (1986) Regulation of bovine sperm motility and cyclic adenosine 3′,5′-monophosphate by adenosine and its analogues. *Biol Reprod* **34:**468–477.
Wang J, Schaner ME, Thomassen S, Su SF, Piquette-Miller M, and Giacomini KM (1997) Functional and molecular characteristics of Na(+)-dependent nucleoside transporters. *Pharm Res (NY)* **14:**1524–1532.
Ward JL, Sherali A, Mo ZP, and Tse CM (2000) Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. *J Biol Chem* **275:**8375–8381.
Yamaoka K, Tanigawara Y, Nakagawa T, and Uno T (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. *J Pharmacobiodyn* **4:**879–885.
Yao SY, Ng AM, Muzyka WR, Griffiths M, Cass CE, Baldwin SA, and Young JD (1997) Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. *J Biol Chem* **272:**28423–28430.

Address correspondence to: Dr. Ikumi Tamai, Department of Molecular Biopharmaceutics, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamasaki, Noda, Chiba, 278-8510, Japan. E-mail: tamai@rs.noda.tus.ac.jp