molecular pharmaceutics

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Functional Characterization of Apical Transporters Expressed in Rat Proximal Tubular Cells (PTCs) in Primary Culture

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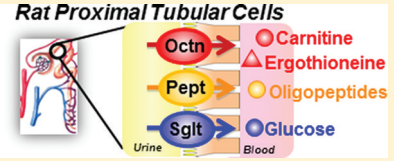
Supporting Information

ABSTRACT: Since *in vitro* cell culture models often show altered apical transporter expression, they are not necessarily suitable for the analysis of renal transport processes. Therefore, we aimed here to investigate the usefulness of primary-cultured rat proximal tubular cells (PTCs) for this purpose. After isolation of renal cortical cells from rat kidneys, PTCs were enriched and the gene expression and function of apical transporters were analyzed by means of microarray, RT-PCR and uptake experiments. RT-PCR confirmed that the major apical transporters were expressed in rat PTCs. Na^+-dependent uptake of α-methyl-D-glucopyranoside (αMG), ergothioneine and carnitine by the PTCs suggests functional expression of Sglts, Octn1 and Octn2, respectively. Inhibition of pH-dependent glycylsarcosine uptake by low concentration of cephalexin, which is a β-lactam antibiotics recognized by Pepts, indicates a predominant role of high affinity type Pept2, but not low affinity type Pept1, in the PTCs. Moreover, the permeability ratio of [^{14}C]αMG (apical to basolateral/basolateral to apical) across PTCs was 4.3, suggesting that Sglt-mediated reabsorptive transport is characterized. In conclusion, our results indicate that rat PTCs in primary culture are found to be a promising *in vitro* model to evaluate reabsorption processes mediated at least by Sglts, Pept2, Octn1 and Octn2.



KEYWORDS: *proximal tubules, kidney, renal reabsorption, SLC transporter, ABC transporter*

INTRODUCTION

Renal plasma membrane transporters play a role in maintaining nutrient homeostasis by reabsorbing various nutrients across the apical membranes of proximal tubules.[1] Hexoses and protein degradation products (e.g., oligopeptides) are known to be reabsorbed via apically expressed concentrative transporters, Na^+-dependent glucose transporters (SGLT1 and SGLT2) and H^+-cotransporter PEPT2,[2] in proximal tubules, particularly in the S_1 segment. We have shown that L-carnitine, an essential substance for β-oxidation of fatty acids, is reabsorbed via organic cation/carnitine transporter OCTN2.[3,4] Absorption of many other nutrients is regulated by various transporters expressed in proximal tubular cells (PTCs).[5−7] On the other hand, secretion of electrically charged compounds from tubular cells is mediated by other transporters, including organic cation[8] and anion[9,10] transporters, and the ATP-driven multidrug resistance transporters.[7,11] Therefore, functional expression of renal transporters in PTCs could be a determinant of urinary excretion rate, residence time within the body, and plasma concentrations of drugs in clinical use as well as nutrients. Although kidney slices are useful for *in vitro* evaluation of basolateral transport, currently available experimental models for apical transport suffer from various disadvantages. To date, membrane transport of various substances across the apical membranes of the proximal tubular cells (PTCs) has been examined using several cultured cell models, e.g., human PTC HK-2,[12] HKC and Caki-1 cells,[13−15] and rat PTC NRK-52E cells.[16] These artificially immortalized cell lines are technically useful and convenient. They, however, are not necessarily appropriate to assess physiological reabsorption processes, because the expression levels of transporters responsible for renal disposition of nutrients and drugs have been altered in these cells, as compared with those of native kidney, freshly isolated tissues or primary-cultured PTCs.[16−18] Therefore, it remains important to establish an appropriate cell culture model for evaluation of renal disposition of solutes.

It has been reported that primary cultures of human PTCs are structurally polarized, with numerous microvilli and tight junctions at the apical side, and preserve various characteristic features of proximal tubules, e.g., enzyme activities of alkaline phosphatase and γ-glutamyl-transferase.[19,20] Therefore, primary-cultured PTCs are thought to be a possible candidate of an *in vitro* model for studies of the mechanisms of renal apical membrane transport, especially those involved in reabsorption. In this work, rats were chosen as a source of PTCs because they are often used in preclinical studies for drug development. We present functional properties of rat PTCs in primary culture with alterations in transporter gene expression

Received: February 28, 2011
Revised: September 20, 2011
Accepted: October 16, 2011
Published: October 17, 2011

during primary culture based on microarray analysis, indicating that the primary-cultured PTC is promising to evaluate the reabsorption processes mediated by Sglts, Pept2, Octn1 and Octn2.

EXPERIMENTAL SECTION

Materials. [^{3}H]Ergothioneine (3.7 GBq/mmol, custom-made), [^{14}C]methyl-α-D-glucopyranoside (αMG, 50 Ci/mmol), [^{3}H]glycylsarcosine (Gly-Sar, 0.5 Ci/mmol) and [^{3}H]mitoxantrone (2.3 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). L-[Methyl-^{3}H]-carnitine (80 Ci/mmol) and [carboxyl-^{14}C]inulin carboxyl (2.5 mCi/g) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). [^{3}H]Digoxin and [^{3}H]estrone 3-sulfate were obtained from PerkinElmer Life Sciences (Waltham, MA). Percoll and collagenase (type I) were purchased from Sigma-Aldrich (St. Louis, MO) and Wako Pure Chemicals (Osaka, Japan), respectively. All other reagents were of the highest grade available from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemicals (Osaka, Japan), Kanto Chemicals (Tokyo, Japan), and Nacalai Tesque (Kyoto, Japan).

Isolation of Rat PTCs and Cell Culture. PTCs were isolated based on the previous report[21] with several modifications. Prior to surgery, all glassware and surgical tools were sterilized in an autoclave, and the abdomens of rats were shaved and cleansed with 70% (v/v) ethanol. After surgical excision of the kidneys, renal cortical cells were obtained by collagenase perfusion. In general, kidneys were perfused with Ca^{2+}-free Hanks buffer (118 mM NaCl, 4.8 mM KCl, 0.96 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 25 mM HEPES, 2% (w/v) BSA, pH 7.4) containing 0.5 mM EGTA at a flow rate of 8 mL/min for 10 min, followed by perfusion with Hanks buffer containing 0.23% (wt/vol) collagenase (type I) and 2 mM $CaCl_2$ for 15−18 min at a flow rate of 5 mL/min. All buffers were bubbled with oxygen gas and maintained at 37 °C during perfusion. The collagenase used was approximately 192 units/mg dry weight. At the end of collagenase perfusion, cells released from kidney were collected into Krebs−Henseleit buffer (2.55 mM $CaCl_2$, 25 mM HEPES, and 2% (w/v) BSA, pH 7.4). To obtain enriched fractions of renal PTCs, cortical cells (1.25 mL) were layered on 8.75 mL of an isosmotic Percoll solution (118 mM NaCl, 4.8 mM KCl, 0.96 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.28 mM $CaCl_2$, 25 mM $NaHCO_3$, 25 mM HEPES, 1% (w/v) BSA, 45% (v/v) Percoll, pH 7.4) in 15 mL polycarbonate centrifuge tubes and centrifuged for 30 min at 20000g. PTCs stayed in the upper layer, which contained cells derived from both convoluted and straight segments of the proximal tubules. Then, the freshly isolated cells were suspended in Krebs−Henseleit buffer to give a concentration of 1.0×10^5 cells/mL. For primary cultures, the obtained PTCs were seeded at a density of 0.5×10^5 cells/well on 24-well collagen-coated polystyrene tissue culture dishes (Iwaki, Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's medium/F12+GlutaMAX medium (Invitrogen Corp., Carlsbad, CA) with 10% fetal bovine serum (Hyclone; Logan, UT), 100 U/mL of penicillin and 100 mg/mL of streptomycin, and 1 mM sodium pyruvate. The medium was changed on days 1, 3, 5, and 7 after initial seeding. Cultures were grown at 37 °C in a humidified incubator under an atmosphere of 95% air, 5% CO_2.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was prepared from primary-cultured PTCs using ISOGEN (Nippon Gene, Toyama, Japan), and then reverse-transcribed to cDNA with Im-Prom-IITM reverse transcriptase (Promega, Madison, WI, USA) using an oligo(dT) primer (Invitrogen). mRNA expression of transporters was detected by RT-PCR with the respective gene specific primers shown in Table 1. PCR was performed for 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s. PCR products were analyzed by electrophoresis in 2% agarose gel and visualized with ethidium bromide.

Table 1. Oligonucleotide Primers Used for RT-PCR

	primer	
transporter name	forward (5′ → 3′)	reverse (5′ → 3′)
Sglt1	atggacagtagcaccttgagcc	tagccccagagaagatgtctgc
Sglt2	cattgtctcaggctggtactgg	ggacactgccacaatgaacacc
Glut1	tcttccaactcaaccaacca	atacacagcagggcaggagt
Glut2	ttggctttcactgtcttcactg	gaaccagtcctgaaattagccc
Oat1	agagtcacagagccctgcat	gcccaggctgtagacatagc
Oat2	cgctcagaattctcctccac	acatccagccactccaactc
Oat3	tcctggtgggtaccagagtc	ctgcatttctgaaggcacaa
Oct1	gatctttatcccgcatgagc	cttctgggaatcctccaagt
Oct2	catcgaggatgccgagaa	acagaccgtgcaagctac
Oct3	tcagagttgtacccaacgacatt	tctgccacactgatgcaact
Octn1	acctcagtgggttactttgctc	ctccgctgtgaagacgtaca
Octn2	tttcgtgggtgtgctgat	gtggaaggcgcaacaatcccatt
Pept1	gtgtggggccccaatctataccgt	gtttgtctgtgagacaggttccaa
Pept2	cctccaaagaagtcacctccg	cacatctcctctcagcatggg
Urat1	tgacaccgagccatgtgag	ggattccagccaggaagatg

Uptake Study by Rat PTCs. For uptake study of various radiolabeled substrates, primary-cultured PTCs were plated onto 24-well collagen-coated tissue culture plates at a density of 0.5×10^5 cells/well. The cells were cultured for 5 days, then washed three times with ice-cold transport medium (130 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.4) before uptake assay of radiolabeled substrates, except in the case of [^{3}H]Gly-Sar, for which the medium pH was 6.0. In the case of αMG uptake assay, D-glucose was removed from the transport medium. Uptake reaction was initiated by adding transport medium containing a radiolabeled substrate to each well, and incubation was continued at 37 °C for a certain period of time. In inhibition studies, each inhibitor was added simultaneously with the radiolabeled substrate. The uptake was terminated by addition of ice-cold transport medium, and then the cells were washed three times with the medium to remove adhering substrate, and lysed with 0.2 mL of 1 M NaOH. Finally, the lysate was neutralized with HCl.

Transcellular Transport Experiments for [^{14}C]αMG. For transcellular transport experiments, PTCs were seeded on 12-well Transwell plates (pore size, 3.0 μM) at a density of 0.5×10^5 cells/well. The cells were cultured for 5 days, then washed three times with transport medium (130 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES, pH 7.4), and preincubated in the medium at 37 °C for 10 min. The uptake reaction was initiated by adding transport medium containing a radiolabeled substrate to cells.

Statistical Analysis. Radioactivity was quantified with a liquid scintillation counter (Aloka, Tokyo, Japan). For protein assay, cellular protein content was measured with a protein assay kit (Bio-Rad) using BSA (Fraction V) as a standard.[22]

Uptake of all substrates was evaluated after correction for cell surface-associated water content by subtraction of apparent [^{14}C]inulin uptake. To calculate kinetic parameters for carrier-mediated uptake of each substrate tested, uptake rate was fitted to eq 1 by means of a nonlinear least-squares regression analysis using Kaleidagraph (Synergy Software, Reading, PA):

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

where v, s, K_m, and V_{max} are the uptake rate of substrate (pmol per indicated time per mg of protein), the substrate concentration in the medium (μmol/L), the apparent Michaelis–Menten constant (μmol/L), and the maximal uptake rate (pmol per indicated time per mg of protein), respectively. Statistical significance of differences was determined with Student's t test, and a P value of less than 0.05 was considered statistically significant.

RESULTS

Cell Morphology. First of all, the morphological characteristics of PTCs in primary culture were examined under a conventional inverted light microscope. PTCs became confluent about 5 days after isolation. The monolayer of PTCs showed a typical epithelial morphology with cobblestone-like cells in the center of the cell islands. PTCs appeared connected one to another (Figure 1), suggesting that they form tight junctions. This morphology is typical of proximal tubular epithelial cells.[21]

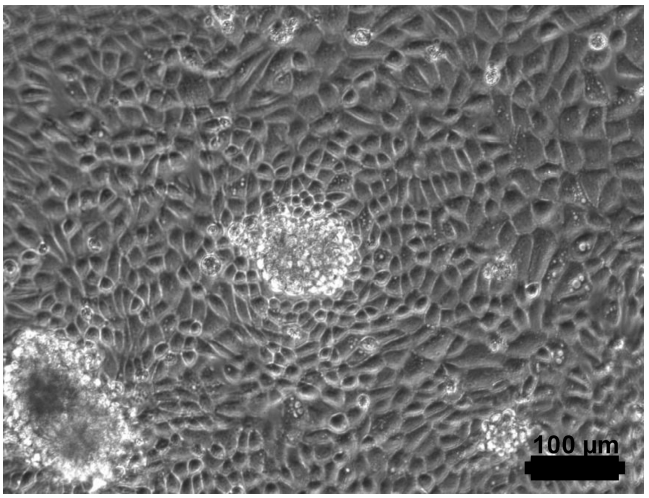


Figure 1. Morphology of primary-cultured PTCs. Photograph of primary-cultured PTCs on day 5, taken under a standard phase-contrast light microscope. The black bar at the right bottom corner indicates 100 μm.

mRNA Expression of Transporter Genes in PTCs in Primary Culture. In order to examine changes of SLC transporter gene expression during primary culture, we employed a rat DNA microarray analysis for whole kidney tissue, freshly isolated tubular cells (day 0) and cultured PTCs on day 1 and day 5. Supplemental Tables 1 and 2 in the Supporting Information show fold changes in mRNA expression of 72 SLC and 28 ABC transporter genes that may be involved in drug reabsorption. Numbers of SLC and ABC transporter genes, whose fold change normalized to that of the whole kidney tissue is less than 0.5, were 45 (62.5%) and 18 (64.3%), respectively, in primary-cultured PTC on day 5. No significant correlation in fold changes between the freshly isolated and PTC (day 5) was observed, but fold changes correlated well ($R > 0.90$) between cultured cells for 1 and 5 days, suggesting that the transporter gene expression profile is unlikely affected by culture time. First of all, function of major ABC transporters was evaluated by measuring intracellular accumulation of digoxin (for P-glycoprotein) and mitoxantrone (for Bcrp1) in the presence of 10 μM elacridar, an inhibitor for both P-glycoprotein and mitoxantrone, as well as estrone 3-sulfate in the presence of 50 μM MK-571, an inhibitor specific to multidrug resistance associated proteins (Mrp). There was no significant increase in intracellular accumulation of each substrate with a respective inhibitors (Supplemental Figure 1 in the Supporting Information). Therefore, it is not considered that ABC transporters affect influx of other substrates used, and SLC transporters responsible for reabsorption of slutes in proximal tubules are further studied.

The mRNA expression of physiologically and pharmacologically important SLC transporters was examined by RT-PCR. Figure 2 displays mRNA expression patterns of transporter

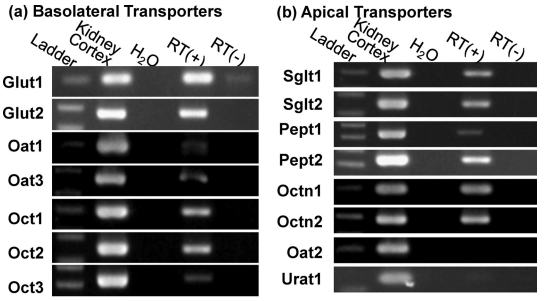


Figure 2. RT-PCR analysis of transporter gene expression in kidney cortex and primary-cultured PTCs. RT-PCR analysis showing PCR products of a variety of transporters expressed in rat kidney cortex and PTCs in primary culture (day 4). Panel (a) shows PCR products for transporters expressed in the basolateral membrane and (b) for those expressed in the apical membrane in PTCs. On day 0, PTCs were prepared and plated on a 24-well tissue culture plate, and total cellular RNA was extracted on day 4. PCR was performed as described in the Experimental Section after (RT+) and before (RT−) reverse transcription. As positive and negative controls, RT-PCR was done with total RNA from rat kidney cortex and without any RNA (RT product was replaced with an equal volume of H_2O).

genes in PTCs cultured for 5 days and in kidney cortical tissues. Na^+-dependent glucose transporters (Sglt1 and Sglt2), organic cation transporters (Oct1, Oct2, Oct3, Octn1 and Octn2), organic anion transporters (Oat1 and Oat3) and H^+-oligopeptide transporters (Pept1 and Pept2) were expressed at the mRNA level in primary-cultured PTCs. Microarray analysis (Supplemental Table 1 in the Supporting Information) indicates that Na^+-independent facilitative glucose transporter 1 (Glut1) expression was increased 4.3-fold in PTCs on day 5 as compared to that in kidney tissue, but expression of the rest of transporter genes shown in Figure 2, except for Oct3, was decreased. Among transporters with decreased expression, the levels of Glut2 (2.35-fold), Sglt1 (4.54-fold), Octn1 (10.1-fold) and Octn2 (12.5-fold) mRNAs were relatively well maintained in primary-cultured PTCs on day 5. mRNA expression of Oat2 and uric acid transporter (Urat1) was not detected in primary-cultured PTCs.

Activity of Transporters Expressed in PTCs in Freshly Isolated Suspension Culture. Gene expressions of SLC

transporters are more likely preserved in the freshly isolated tubular cells than in the primary-cultured PTCs (Supplemental Table 1 in the Supporting Information). At first, in order to evaluate functional expression of major apical transporters, Sglt, Pept, Octn1 and Octn2, uptake of test compounds that undergo reabsorption at PTCs including [^{14}C]αMG (a nonmetabolized substrate for Sglts), [^{3}H]Gly-Sar (for Pepts), [^{3}H]ergothioneine (for Octn1) and [^{3}H]carnitine (for Octns2), respectively, were examined in freshly isolated tubular cells in suspension culture. Uptake of [^{14}C]αMG and [^{3}H]carnitine was Na^+ ion dependent, and decreased to approximately 40% and 50% in the absence of Na^+, compared to the control (the presence of Na^+). However, both Na^+-dependent [^{3}H]-ergothioneine uptake and H^+-dependent [^{3}H]Gly-Sar uptake were not observed at all (data not shown). Furthermore, [^{14}C]uric acid uptake was not inhibited by a well-established inhibitor of URAT1, benzbromarone, even at 100 μM (data not shown), although the expression of URAT1 was preserved according to the microarray analysis (Supplemental Table 1 in the Supporting Information).

Activity of Transporters Expressed in PTCs in Primary Culture. Uptake of [^{14}C]αMG increased linearly over 20 min in primary-cultured PTCs in the presence of Na^+, while the uptake was completely abolished in the absence of Na^+ (Figure 3a). Phlorizin, a potent inhibitor of Sglts, but not facilitative glucose transporters (e.g., Gluts), significantly reduced the uptake of [^{14}C]αMG at 10 min to about 3% of the control uptake measured in its absence (Figure 3b). In order to determine which Sglt, Sglt1 or Sglt2, is involved, we examined the effect of D-galactose, which is recognized by Sglt1 but not by Sglt2,[23,24] on the [^{14}C]αMG uptake. Even at high concentration (1 mM), D-galactose did not affect the [^{14}C]αMG uptake, suggesting that Sglt2 is predominantly responsible for the apical uptake of glucose in PTCs in primary culture.

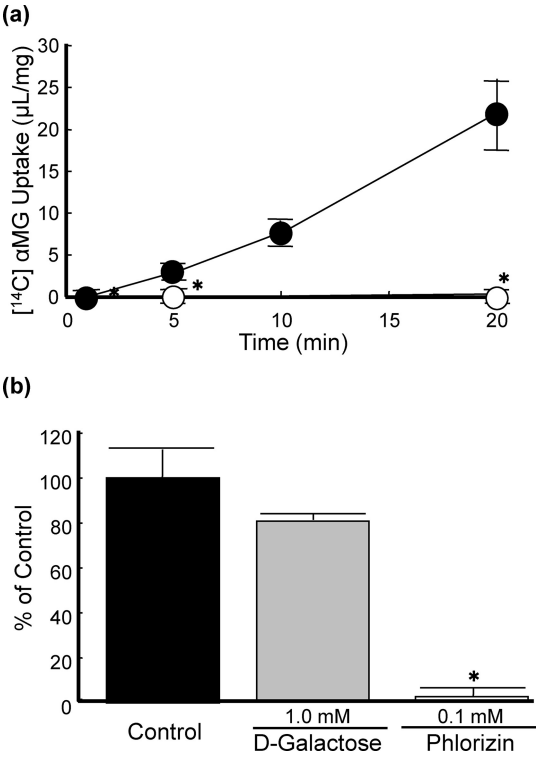


Figure 3. [^{14}C]αMG uptake by primary-cultured PTCs. (a) Time course of [^{14}C]αMG (20 μM) uptake by primary-cultured PTCs. Uptake was measured at pH 7.4 with (closed symbols for control) or without (open symbols) Na^+ for 20 min. (b) Effect of Sglts inhibitors on [^{14}C]αMG uptake by primary-cultured PTCs. Uptake of [^{14}C]αMG (20 μM) was measured at pH 7.4 and 37 °C for 10 min with (D-galactose (grey column), or phlorizin (white column), or without (closed column; control) inhibitors. Each point or bar represents the mean value with SEM (n = 3) of uptake after subtraction of [^{14}C]inulin uptake. * indicates a statistically significant difference from the control by Student's t test ($p < 0.05$).

Similarly, [^{3}H]Gly-Sar uptake was studied in primary-cultured PTCs. Gly-Sar is an N-methylated dipeptide resistant to hydrolysis by peptidase and a well-established substrate of both Pept1 and Pept2. Since [^{3}H]Gly-Sar uptake increased linearly for up to 20 min (Figure 4a), we measured the initial uptake rate at 10 min in the following studies. [^{3}H]Gly-Sar uptake by PTCs was the highest at pH 6.0 (Figure 4b). The [^{3}H]Gly-Sar uptake at pH 6.0 was reduced to 30 and 20% in the presence of 0.5 mM and 10 mM β-lactam antibiotics, cephalexin, that functions as an inhibitor for both Pept1 and Pept2 but shows higher affinity to Pept2 (Figure 4c).

Uptake of an Octn1 substrate, [^{3}H]ergothioneine, was dependent on temperature (Figure 5a). The uptake of [^{3}H]ergothioneine was decreased to 25% and 30% in the absence of Na^+ and in acidic transport medium (pH 6.0), respectively (Figure 5b). Uptake of [^{3}H]ergothioneine by primary-cultured PTCs was saturated at concentrations over 200 μM (Figure 5c) and consisted of a single component with K_m of 7.4 ± 9.3 μM and V_{max} of 71.7 ± 21.1 pmol/mg of protein/5 min (Figure 5c). Furthermore, [^{3}H]carnitine uptake was studied to evaluate functional expression of Octn2. [^{3}H]Carnitine uptake increased linearly over 10 min in primary-cultured PTCs (Figure 6a). [^{3}H]Carnitine uptake decreased to 65% in the absence of Na^+. In addition, [^{3}H]carnitine uptake was reduced by 65% and 48% in the presence of excess amounts of unlabeled carnitine (500 μM) and arginine (500 μM), respectively, suggesting that Octn2 is involved in carnitine uptake by PTCs (Figure 6b).

Transcellular Transport of [^{14}C]αMG in Primary-Cultured PTCs. To determine whether primary-cultured PTCs are suitable for evaluation of transcellular transport, we examined the trans-epithelial electrical resistance (TEER) of PTCs cultured on a Transwell insert. At day 6 after seeding, TEER reached a plateau and remained constant at about 140 $\Omega \cdot cm^2$ in culture for up to a further 6 days (data not shown). Then, the transcellular permeability of [^{14}C]αMG in primary cultures of PTCs on Transwells was evaluated. Apical (AP) to basolateral (BL) (Figure 7a) and BL to AP (Figure 7b) transcellular transport of [^{14}C]αMG across a monolayer of PTCs was measured for 30 min. The AP to BL and the BL to AP permeability values of [^{14}C]αMG were 10.7 ± 0.39, and 2.46 ± 0.03 ($\times 10^{-5}$ cm/s), respectively (Table 2). These rates were significantly higher than paracellular leakage of [^{3}H]-mannitol (1.38 ± 0.29 $\times 10^{-5}$ cm/s). The AP-to-BL permeability of [^{14}C]αMG was significantly reduced in the absence of Na^+ (Table 2). The AP-to-BL permeability ratio of [^{14}C]αMG in transporter buffer containing Na^+ over that without Na^+ was 1.58, whereas the corresponding ratio for BL-to-AP permeability was 0.53. Furthermore, the permeability ratio of [^{14}C]αMG determined as the value of (AP-to-BL)/(BL-to-AP) in the presence of Na^+ was 2.97-fold greater than that in the absence of Na^+. These results demonstrate that the

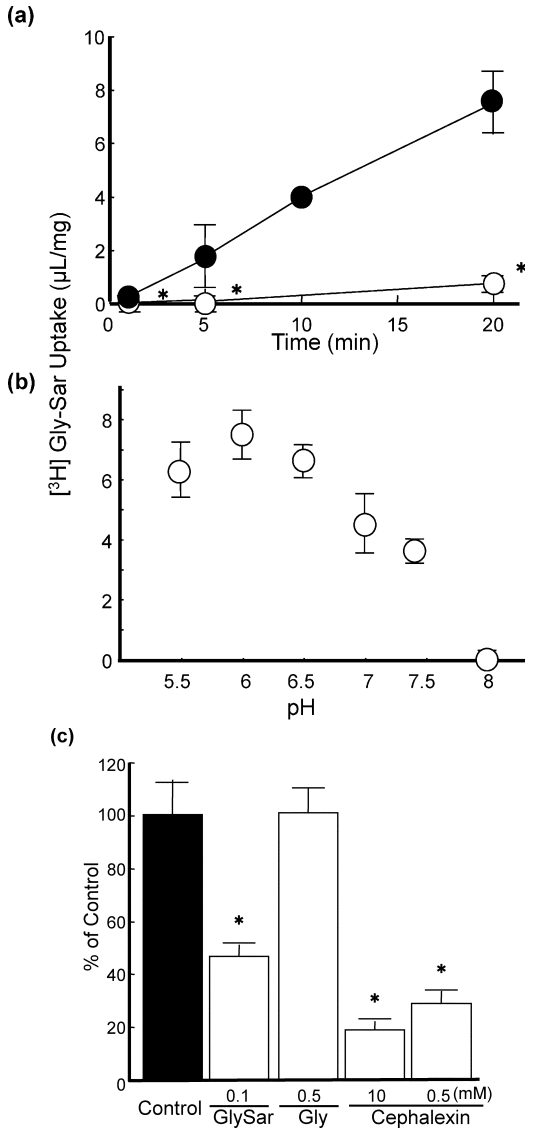


Figure 4. [^{3}H]Gly-Sar uptake by primary-cultured PTCs. (a) Time course of [^{3}H]Gly-Sar (20 μM) uptake by primary-cultured PTCs. Uptake was measured at pH 7.4 and 37 °C (closed column; control) or 4 °C (open column) for 20 min. (b) pH dependence of [^{3}H]Gly-Sar uptake by primary-cultured PTCs was measured. Uptake of [^{3}H]Gly-Sar (1 μM) was measured at various pH values (range 5.5–8.0) for 10 min at 37 °C. (c) Inhibitory effect of amino acid, dipeptide and β-lactam antibiotics on [^{3}H]Gly-Sar uptake by primary-cultured PTCs. Uptake of [^{3}H]Gly-Sar (1 μM) by primary-cultured PTCs was measured at pH 7.4 and 37 °C for 10 min with (open column) or without (closed column; control) inhibitors. Each point or bar represents the mean value with SEM (n = 3) after subtraction of [^{14}C]inulin uptake. * indicates a statistically significant difference from the control by Student's t test ($p < 0.05$).

present cultured cell model adequately reflects Sglt function in AP to BL transport.

DISCUSSION

There is an increasing demand for methods to evaluate the activities of transporters responsible for renal reabsorption of drugs and nutrients in order to predict *in vivo* renal disposition. Apparent activity of transporters in the basolateral membrane can be measured by using kidney slices, but an effective method for measuring the activities of apical membrane transporters has

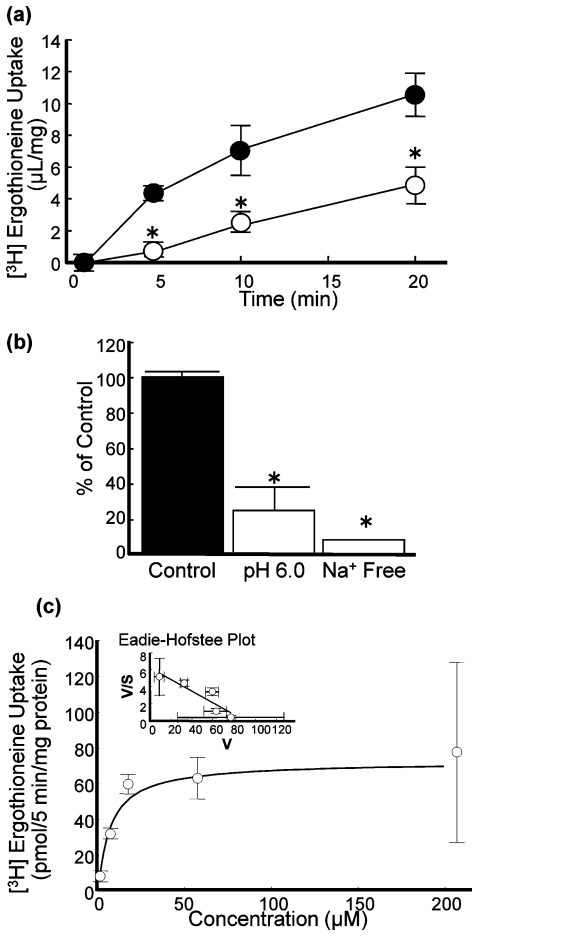


Figure 5. [^{3}H]Ergothioneine uptake by primary-cultured PTCs. (a) Time course of [^{3}H]ergothioneine (5 μM) uptake by primary-cultured PTCs. Uptake was measured at pH 7.4 and 37 °C (closed column; control) or 4 °C (open column) for 20 min. (b) Effect of Na^+ and pH on [^{3}H]ergothioneine uptake by primary-cultured PTCs. Uptake of [^{3}H]ergothioneine by primary-cultured PTCs was measured at pH 7.4 and 37 °C for 5 min. (c) Concentration dependence of [^{3}H]-ergothioneine uptake by primary-cultured PTCs. The data were fitted to the Michaelis–Menten equation by a nonlinear least-squares regression analysis, as described in the Experimental Section. Inset: The uptake was analyzed by means of the Eadie–Hofstee plot. Each point or bar represents the mean ± SEM (n = 3), after subtraction of the uptake of [^{14}C]inulin.

not been established. In the present study, PTCs cultured on plastic or semipermeable membranes were assessed the aptitude for this purpose, and we were able to show that several important apical uptake transporters are functionally maintained in these cells. Species differences, to date, have been reported in transporter expression between humans and rats. For example, hOCT1 is little expressed, whereas significant expression of rOct1 is detected in the basolateral membranes.[25] In the case of OAT2, it is expressed at the basolateral membranes and plays a role in elimination of anionic compounds from blood to PTCs in humans,[26] while rOat2 was detected in the apical surface of the tubules in the medullary thick ascending limb of Henle's loop,[27] and may be contributable to reabsorption of the compounds. Despite such species differences, this PTC model cell can be usable to examine activities of the common transporters involved in reabsorption in both species. Hence, Sglts, Pepts, Octn1/2 and

dx.doi.org/10.1021/mp200096r | Mol. Pharmaceutics 2011, 8, 2142–2150

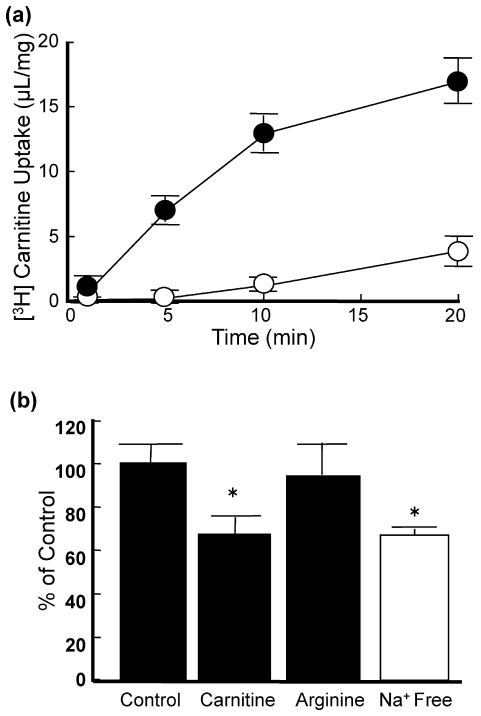


Figure 6. [^{3}H]Carnitine uptake by primary-cultured PTCs. (a) Time course of [^{3}H]carnitine uptake by primary-cultured PTCs. Uptake was measured at pH 7.4 and 37 °C (closed column; control) or 4 °C (open column) for 20 min. (b) Effect of Na^+ and inhibitors on [^{3}H]carnitine uptake by primary-cultured PTCs. Uptake of [^{3}H]-carnitine (12 nM) by primary-cultured PTCs was measured at 37 °C for 10 min with (closed column) or without (open column) Na^+. Each point or bar represents the mean ± SEM (n = 3) after subtraction of the uptake of [^{14}C]inulin. * indicates a statistically significant difference from the control (with Na^+ and without inhibitors) by Student's t test ($p < 0.05$).

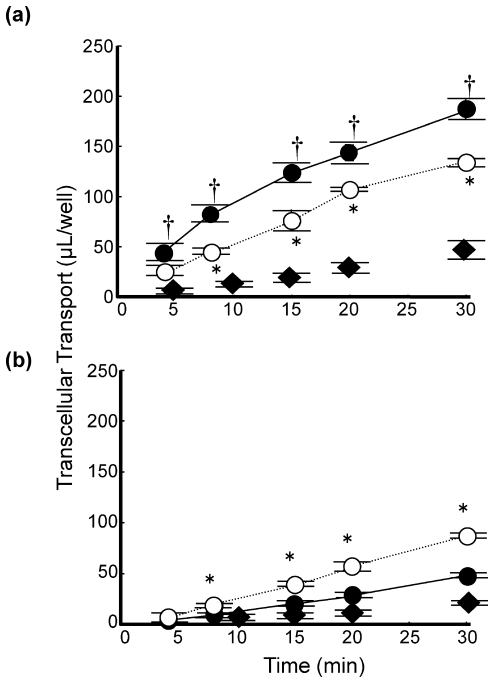


Figure 7. Effect of Na^+ on [^{14}C]αMG trans-epithelial transport across primary-cultured PTC monolayer. (a) Transcellular transport (in the AP to BA direction) and (b) transcellular transport (in the BA to AP direction) of [^{14}C]αMG and [^{3}H]mannitol. [^{14}C]αMG was added to the apical side of a primary-cultured PTC monolayer with (closed symbol, control) or without (open symbol) Na^+. Closed diamonds show transcellular transport of [^{3}H]mannitol as a paracellular marker. For 30 min at 37 °C, [^{14}C]αMG and [^{3}H]mannitol translocated into the basolateral side (a) and the apical side (b) were measured. Their apparent permeability coefficients were calculated as described in the Experimental Section (Table 2). Each point represents the mean ± SEM (n = 3). * and † indicate a statistically significant difference in transcellular transport in Na^+-free buffer vs that in the control (with Na^+), and in transcellular transport in the direction of AP to BA vs BA to AP, measured in the buffer with Na^+, by Student's t test ($p < 0.05$), respectively.

Urat1 were chosen for the assay in the present study because species difference has not been seen so far.

We initially examined changes of mRNA expression by employing a rat DNA microarray assay. In many genes, mRNA expression seemed to be preserved in freshly isolated PTCs (day 0). However, function of transporters for ergothioneine, uric acid and oligopeptides was unlikely consistent to their reabsorption literally described. Since freshly isolated cells did not attach well to plastic plates, uptake had to be done in suspension culture of the PTCs. Therefore, we were not able to distinguish apical from basolateral uptake of the test compounds. That led us to culture PTCs on the plates for days. mRNA expression of major transporters expressed in PTCs, but Oat2 and Urat1, were detected under the culture conditions (Figure 2). To date, the absence of gene expression of transporters has been reported in several proximal tubule-derived mammalian cell culture models. For example, Caki-1 cells lack mRNA expression of organic cation transporters, including OCT1, OCT2 and OCTN1, and organic anion transporters, including OAT1, OAT2 and OAT3.[15,28] Moreover, Oat1, Oat2, Oat3, Oatp1a1 and Oatp1a3 were not detected in NRK-52E cells, a rat proximal tubular cell line, in RT-PCR assay.[16] Swine PTCs, LLC-PK1, which are often used to evaluate drug transport through proximal tubular cells, also lack several organic anion transporters[18] and proximal brush-border enzymes, compared with those in isolated and primary-cultured PTCs.[17] Taking these findings together, it is considered that expression of major transporters was relatively well preserved in the primary-cultured PTCs. Since previous reports showed that medium composition might influence the expression and activity of brush-border enzymes,[29,30] manipulating medium composition may help to understand alteration of gene expression in primary-cultured PTCs. Future study is warranted to compare protein expression levels with functional activity in the primary-cultured PTCs to accurately describe the usefulness of this cell model.

Table 2. Effect of Na^+ on [^{14}C]αMG Permeability across Primary-Cultured PTC Monolayer[a]

	control	Na^+-free	control/Na^+-free
A to B	10.68 ± 0.39	6.76 ± 1.05	1.58
B to A	2.46 ± 0.03	4.64 ± 0.23	0.53
A to B/B to A	4.34	1.46	

[a]Each value is the mean ± SEM (×10^{-5} cm/s). [^{14}C]αMG (2.5 μM) was added to the apical side of primary-cultured PTC monolayer with or without Na^+. After 30 min at 37 °C, [^{14}C]αMG (2.5 μM) transported into basal side was measured. † indicates a statistically significant difference (AP-to-BL vs. BL-to-AP). * indicates a statistically significant difference (control vs Na^+-free).

dx.doi.org/10.1021/mp200096r | Mol. Pharmaceutics 2011, 8, 2142–2150

Uptake studies with a variety of endogenous substrates were conducted (Figures 3 through 6). It has been generally accepted that uptake reflects reabsorption processes since *p*-aminohippuric acid by PTCs plated on culture dishes was shown to correspond to its reabsorption from the apical lumen of proximal tubules.[31] In the proximal tubules, most of glucose filtrated by glomerulus is reabsorbed via Sglts, which are essential to maintain the plasma glucose level. Both Sglt1 (high affinity/low capacity) and Sglt2 (low affinity/high capacity) are expressed in the brush-border membranes in PTCs.[23,24] Although phlorizin is a common inhibitor of both Sglts, they differ in binding affinity for D-galactose; i.e., D-galactose preferentially inhibits Sglt1 over Sglt2.[23,24] As shown in Figure 3b, the finding that αMG uptake was inhibited by phlorizin but not by D-galactose implies that Sglt2 more dominantly contributes to reabsorption of glucose. These results agree with the previous reports of phlorizin-sensitive Na^+-dependent glucose transport at the apical side in PTCs.[32]

Pept1 and Pept2 are pH-dependent peptide transporters, and Pept2 is predominantly expressed in the brush-border membranes of PTCs.[2] Figure 4 suggests that Pepts are functional, because [^{3}H]Gly-Sar uptake by PTCs was induced by an inward H^+ gradient. Cephalexin has greater affinity to Pept2 with a K_i (inhibition constant) of 0.049 mM (the K_i of Pept1 is approximately 4.5 mM).[33] Therefore, the 70% reduction of [^{3}H]Gly-Sar uptake by cephalexin at the concentration of 0.5 mM is accounted for by its competitive inhibition of high-affinity-type Pept2, but not low-affinity-type Pept1, implying that this model reflects well the *in vivo* oligopeptide reabsorption via Pept2 at the proximal tubules.

We have originally identified Octn1 as a pH-dependent organic cation transporter expressed in PTCs.[18] Octn1 has been reported to transport ergothioneine in a Na^+ and pH-dependent manner.[34,35] Since Octn1 is localized on the apical membrane of PTCs,[36] we examined whether Octn1 function was retained in primary-cultured PTCs. Na^+- and pH-dependent ergothioneine uptake was observed (Figure 5b). Kinetic analysis of the initial uptake of ergothioneine by rat Octn1 showed a linear Eadie−Hofstee plot with a K_m value of 7.4 μM (Figure 5c), which is close to our previously reported value (4.64 μM),[35] suggesting that Octn1 is functionally expressed at the surface of the primary-cultured PTCs. In addition, Octn2-mediated carnitine transport in the present system was examined, since Octn2 is responsible for reabsorption of carnitine.[3,4,37] Although carnitine is transported by amino acid transporter $ATB^{0,+}$ in an Na^+-dependent manner,[38] our data show that carnitine uptake by PTCs was not inhibited by arginine, a typical substrate of $ATB^{0,+}$ (Figure 6b). Therefore, it is considered that carnitine uptake in PTCs in primary culture reflects Octn2 function. However, carnitine uptake was not completely abolished by removal of Na^+ (Figure 6b), hence other transporter system(s) may play a role in carnitine reabsorption.

Finally, we examined whether primary-cultured PTCs can be used to evaluate transcellular transport of small molecules, corresponding to reabsorption. In the presence of Na^+, AP-to-BL permeation of [^{14}C]αMG was higher than BL-to-AP permeation (Table 2). Furthermore, AP-to-BL transport of [^{14}C]αMG in the presence of Na^+ was higher than that in the absence of Na^+, while a substantial amount of AP to BL transport of [^{14}C]αMG was preserved in the Na^+-free condition, compared with paracellular leakage evaluated in terms of the permeability of mannitol. This significant Na^+-independent transport was inconsistent with our results in the uptake experiments (Figure 3a), where [^{14}C]αMG was almost completely taken up in a Na^+-dependent manner. A possible explanation is that Na^+-independent glucose transporter Glut may play a role in AP-to-BL transcellular transport of [^{14}C]αMG, although such a discrepancy between uptake and transport experiments in the PTCs used in the present study should be clarified in detail if this system is to be further developed. The observation that BL-to-AP permeability of [^{14}C]αMG in the absence of Na^+ was greater than that in the presence of Na^+ could be explained by reuptake of permeated [^{14}C]αMG into the AP side by Sglt expressed in the apical membranes, resulting in a decreased appearance of αMG on the apical side, which could not occur in Na^+-free buffer. In the present study, we did not examine transcellular transport of other substrates, and further study needs to be done to characterize transcellular transport for other molecules in PTCs in order to assess the usefulness as a model.

In conclusion, PTCs in primary culture maintained functional expression of major transporters, such as Sglt2, Pept2, Octn1 and Octn 2, involved in renal reabsorption of natural nutrients and drugs, and could be a promising model for evaluation of apical uptake. Further cytohistochemical study for the transporter protein should validate reliability of this *in vitro* cell culture model to evaluate not only renal disposition of drugs, but also drug−drug and drug−food interactions in the renal reabsorption process.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental Table 1 presenting changes of SLC transporter gene expression in PTCs, Supplemental Table 2 presenting changes of ABC transporter gene expression in PTCs, and Supplemental Figure 1 depicting intracellular accumulation of ABC transporter substrates. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

PTC, proximal tubular cells; SLC, solute carrier; ABC, ATP-binding cassette; αMG, α-methyl-D-glucopyranoside; AP, apical; BP, basolateral; SGLT/Sglt, Na^+-dependent glucose transporters; Gly-Sar, glycylsarcosine; RT-PCR, reverse transcription polymerase chain reaction; OCT/Oct, organic cation transporter; OAT, organic anion transporter; PEPT/Pept, H^+-oligopeptide transporter; TEER, trans-epithelial electrical resistance

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