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Transcellular transport of organic cations in double-transfected MDCK cells expressing human organic cation transporters hOCT1/hMATE1 and hOCT2/hMATE1

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ABSTRACT

To clarify the transcellular transport of organic cations via basolateral and apical transporters, we established double-transfected Madin–Darby canine kidney (MDCK) cells expressing both human organic cation transporter hOCT1 and hMATE1 (MDCK-hOCT1/hMATE1), and hOCT2 and hMATE1 (MDCK-hOCT2/hMATE1) as models of human hepatocytes and renal epithelial cells, respectively. Using the specific antibodies, hOCT1 and hMATE1 or hOCT2 and hMATE1 were found to be localized in the basolateral and apical membranes of MDCK-hOCT1/hMATE1 or MDCK-hOCT2/hMATE1 cells, respectively. A representative substrate, [^{14}C]tetraethylammonium, was transported unidirectionally from the basolateral to apical side in these double transfectants. The optimal pH was showed to be 6.5 for the transcellular transport of [^{14}C]tetraethylammonium, when the pH of the incubation medium on the apical side was varied from 5.5 to 8.5. The basolateral-to-apical transport also decreased in the presence of 10 mM 1-methyl-4-phenylpyridinium or 1 mM levofloxacin on the basolateral side of both double transfectants. In MDCK-hOCT2/hMATE1 cell monolayers, but not in MDCK-hOCT1/hMATE1 cell monolayers, the accumulation of [^{14}C]tetraethylammonium was decreased in the presence of 10 mM 1-methyl-4-phenylpyridinium, but significantly increased in the presence of 1 mM levofloxacin. The uptake of [^{14}C]tetraethylammonium, [^{3}H]1-methyl-4-phenylpyridinium, [^{14}C]metformin and [^{3}H]cimetidine, but not of [^{14}C]procainamide and [^{3}H]quinidine, by HEK293 cells was stimulated by expression of the hOCT1, hOCT2 or hMATE1 compared to control cells. However, transcellular transport of [^{14}C]procainamide and [^{3}H]quinidine was clearly observed in both double-transfectants. These cells could be useful for examining the routes by which compounds are eliminated, or predicting transporter-mediated drug interaction.

1. Introduction

Renal tubular secretion of drugs, toxins and endogenous metabolites is one of the most important functions in the kidney. The characteristics of the transport of tetraethylammonium (TEA), a representative substrate of the organic cation transport system, by the basolateral and brush-border membranes revealed that transcellular transport across the renal epithelial cells was mediated by basolateral uptake from blood and subsequent extrusion from the cells into the lumen. The mechanisms of renal secretion of cationic drugs were examined using isolated membrane vesicles from rat kidney

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[1]. It was reported that the TEA transport across basolateral membranes was stimulated by an inside-negative membrane potential, and that across brush-border membranes was driven by an H^+ gradient.

Human organic cation transporter hOCT1 (SLC22A1) and hOCT2 (SLC22A2), which act as membrane potential dependent organic cation transporters, are expressed in the basolateral membranes of the liver and kidney, respectively [2]. The basolateral entry of cationic drugs is mediated mainly by hepatic hOCT1 and renal hOCT2 in humans, and hepatic Oct1 and renal Oct1 and Oct2 in mice, depending on the membrane potential [3–5]. In 2005, human multidrug and toxin extrusion 1 (hMATE1/SLC47A1) was isolated, and hMATE1 is expressed in the liver, kidney and skeletal muscle [6]. Thereafter, we identified a kidney-specific hMATE2-K (SLC47A2) [7]. Both mediated oppositely directed H^+ gradient dependent transported cationic compounds, called as H^+/organic cation antiporter, and were located in the brush-border membranes of the renal proximal tubules. Considering their characteristics, the hMATE family mediated the excretion of cationic drugs from the epithelial cells to luminal side.

Their substrate specificity, membrane localization and driving force suggested that hOCT2 and hMATE1 mediated tubular secretion of cationic drugs from blood to urine [3,7,8]. However, an in vitro model that reflects the vectorial transport of cationic drugs across human epithelial cells has not been established. Consequently, the porcine kidney epithelial cell line LLC-PK_1 has been employed to analyze transcellular transport [9,10]. These studies indicated that cationic drugs were transported unidirectionally from the basolateral to apical side in LLC-PK_1 cell monolayers. Previously, MDCK cells expressing both human organic anion transporter 8 (OATP8/SLCO1B3) and multidrug resistance protein 2 (MRP2/ABCC2) or both human organic anion-transporting polypeptide (OATP-C/SLCO1B1) and MRP2 were constructed to determine the transcellular transport of organic anions, and the vectorial transport of double-transfectants suggested their usefulness as in vitro hepatocyte models with an anion transport system [11,12].

Based on these backgrounds, it is necessary to clarify whether the basolateral hOCT1 or hOCT2 and the apical hMATE1 mediated the transcellular transport of cationic compounds. In the present study, we established MDCK cells stably expressing both hOCT2 and hMATE1 as an in vitro model of human renal epithelial cells. Human hepatocyte model expressing both hOCT1 and hMATE1 was also constructed. Moreover, the availability of these double-transfectants was evaluated to examine the transcellular transport of several cationic drugs.

2. Materials and methods

2.1. Materials

[^{14}C]Tetraethylammonium (TEA; 2.035 GBq/mmol), [^{14}C]creatinine (2.035 GBq/mmol), [^{14}C]procainamide (2.035 GBq/mmol), and [9-^{3}H]quinidine (740 GBq/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). [^{14}C]Metformin (962 MBq/mmol), [^{14}C]guanidine hydrochloride (1.961 GBq/mmol) and [1-^{14}C]-D-mannitol were purchased from Moravek Biochemicals Inc. (Brea, CA). [^{3}H]1-Methyl-4-phenylpyridinium acetate (MPP; 2.7 TBq/mmol) and D-[1-^{3}H(N)]-mannitol were from PerkinElmer Life Analytical Science (Boston, MA). [*N*-Methly-^{3}H]cimetidine (451 GBq/mmol) was from GE Healthcare (Buckinghamshire, UK). All other chemicals used were of the highest purity available.

2.2. Cell culture and transfection

The parental MDCK cells (ATCC CCL-34) obtained from American Type Culture Collection were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The hOCT1 or hOCT2 cDNA was subcloned into the Not I-cut mammalian expression vector pcDNA3.1(+) (Invitrogen). The hMATE1 cDNA was subcloned into the XbaI- and Kpn I-cut mammalian expression vector pcDNA3.1(+)/Hygro (Invitrogen). MDCK cells were cotransfected with either pcDNA3.1(+) containing hOCT1 or hOCT2 cDNA and pcDNA3.1(+)/Hygro containing hMATE1 cDNA using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Forty-eight hours later, the cells split between 1:25 and 1:100 were cultured in complete medium containing G418 (0.5 mg/ml: Nacalai Tesque Inc., Kyoto, Japan) and Hygromycin B (0.2 mg/ml; Invitrogen). Seven to fourteen days after the transfection, single colonies appeared and several G418- and Hygromycin B-resistant colonies were picked out based on the growth rate and morphology of the cells. These MDCK cells were selected on the basis of the cellular uptake of [^{14}C]TEA and named MDCK-vector (MDCK cells cotransfected with pcDNA3.1(+) empty vector and pcDNA3.1(+)/Hygro empty vector), MDCK-hOCT1/hMATE1 (MDCK cells cotransfected with pcDNA3.1(+) containing hOCT1 cDNA and pcDNA3.1(+)/Hygro containing hMATE1 cDNA) and MDCK-hOCT2/hMATE1 (MDCK cells cotransfected with pcDNA3.1(+) containing hOCT2 cDNA and pcDNA3.1(+)/Hygro containing hMATE1 cDNA). For the transcellular transport experiments, cells were seeded on microporous membrane filters [3.0-μm pores, 4.7 (or 1.0) cm^2 growth area] inside a Transwell cell culture chamber (Costar, Cambridge, MA) at a density of 5×10^5 cells/cm^2 with complete medium, as described above. In this study, MDCK cells were used between the 80th and 86th passages. HEK293 cells (American Type Culture Collection CRL-1573) were cultured as well as MDCK cells. pCMV6-XL4 plasmid vector DNA (OriGene Technologies, Rockvill, MD) that contained hOCT1 cDNA or hOCT2 cDNA or pcDNA3.1 (+)/Hygro vector DNA that contained hMATE1 cDNA was introduced into HEK293 cells using LipofectAMINE 2000 Reagent. At 48 h after the transfection, the cells were used for uptake experiment.

2.3. Polyclonal antibodies and immunofluorescence microscopy

Polyclonal antibodies were raised against the candidate peptide as described, previously [7,13]. For immunostaining, the double-transfected MDCK cells were grown 4 days in Matsunami Micro Cover Glass (Matsunami Glass Ind., Ltd., Osaka, Japan). The double-transfected MDCK cells were fixed

2% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 min, and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Potential sites for non-specific antibody binding were blocked by 30 min incubation with 10% FBS and 1 mg/ml RNase A (Nacalai Tesque, Kyoto, Japan) in Dulbecco's modified Eagle's medium. These cells were incubated with primary antibodies (1:200 dilution) specific for hOCT1, hOCT2, or hMATE1 for 1.5 h at room temperature. After washing with PBS, cells were incubated with the secondary antibodies (1:400 dilution) with Alexa-488-labeled phalloidin (Invitrogen) for 30 min at room temperature. All antibodies were diluted in Dulbecco's modified Eagle's medium containing 10% FBS. These cells were examined with a BX-50-FLA fluorescence microscope (Olympus, Tokyo, Japan) at 40× magnification. Images were captured with a DP-50 CCD camera (Olympus) using Studio Lite software (Olympus).

2.4. Measurement of transcellular transport and cellular accumulation

The transcellular transport and cellular accumulation of radiolabeled compounds by the MDCK cells were measured using monolayer cultures grown in Transwell chambers. The incubation medium for the transport experiments contained 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose and 5 mM MES (pH 5.5–6.5) or 5 mM HEPES (pH 7.0–8.5) [7]. The pH of the medium was adjusted with NaOH or HCl. In general, after the culture medium was removed from both sides of the monolayers, cells were incubated for 10 min at 37 °C with 2 ml of incubation medium (pH 7.4) on each side for the 4.7-cm^2 chamber (0.5 ml on the apical side and 1 ml on the basolateral side for the 1.0-cm^2 chamber). The incubation medium was replaced with 2 ml of incubation medium containing radiolabeled compounds on either the apical or basolateral side (1 ml in the basolateral side for the 1.0-cm^2 chamber), unlabeled incubation medium was added to the opposite side. To examine the transcellular transport, an aliquot (100 μl) of the incubation medium in the opposite side was periodically collected. To measure the cellular accumulation, the medium was immediately removed by suction at the end of the incubation period, and the monolayers were rapidly rinsed three times with 2 ml of ice-cold incubation medium (pH 7.4) in each side (1 ml each for the 1.0-cm^2 chamber). The filter was detached from the chambers, and the cells on the filters were solubilized in 0.5 ml of 0.5 N NaOH. The radioactivity of the collected medium (100 μl) and the solubilized cell monolayers (300 μl) was determined in 2 ml or 3 ml of ACSII (GE Healthcare) by liquid scintillation counting. D-[^{3}H]-mannitol or [^{14}C]-D-mannitol was used to calculate paracellular fluxes and extracellular trapping of radiolabeled compounds. The amount of protein in the solubilized cell monolayers was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine γ-globulin as a standard.

2.5. Uptake experiment

Cellular uptake of cationic compounds was measured with HEK293 cells that were grown on poly-D-lysine-coated 24-well plates. The cells were preincubated with 0.2 ml of incubation medium for 10 min at 37 °C. After the removal of the medium, 0.2 ml of incubation medium containing the radiolabeled substrates was added. The medium was aspirated off at the end of the incubation, and the monolayers were rinsed rapidly three times with 1 ml of ice-cold incubation medium. The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in aliquots was determined in 3 ml of ACSII by liquid scintillation counting. For manipulation of the intracellular pH, intracellular acidification was performed by pretreatment with ammonium chloride (30 mM, 20 min at 37 °C, pH 7.4). The protein content of the solubilized cells was determined using a Bio-Rad Protein Assay Kit with bovine γ-globulin as a standard.

2.6. Statistical analysis

Data are expressed as means ± S.E. Data were analyzed statistically using the unpaired Student's *t*-test. Multiple comparisons were performed with Tukey's two-tailed test after a one-way ANOVA. Probability values of less than 0.05 were considered statistically significant.

3. Results

3.1. Expression and localization of hOCT1, hOCT2 and hMATE1 in double-transfected MDCK cells

The expression and localization of hOCT1, hOCT2 and hMATE1 in double transfected MDCK cells were examined by immunofluorescence microscopy. In MDCK-hOCT1/hMATE1 cells, hOCT1 and hMATE1 were localized on the basolateral and apical membrane, respectively (Fig. 1A and B). In MDCK-hOCT2/hMATE1 cells, hOCT2 was localized to the basolateral membrane, in addition to the apical appearance of hMATE1 (Fig. 1C and D). MDCK-vector cells were not observed the expression of hOCT1, hOCT2 and hMATE1 (Fig. 1E–G).

3.2. Transcellular transport and cellular accumulation of TEA by MDCK-vector, MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cells

Fig. 2 shows the transcellular transport and cellular accumulation of [^{14}C]TEA from the basolateral to apical side and the apical to basolateral side by using 4.7-cm^2 chamber. The basolateral-to-apical transport was much greater than the apical-to-basolateral transport, and its rate was nearly constant for up to 60 min in MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cell monolayers (Fig. 2B and C). The accumulation of [^{14}C]TEA from the basolateral side was 66- and 8.4-fold higher than that from the apical side in MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cell monolayers, respectively (Fig. 2E and F). No transcellular transport or cellular accumulation of [^{14}C]TEA was observed in the MDCK-vector cell monolayers (Fig. 2A and D).

3.3. Effect of apical pH on the transcellular transport and cellular accumulation of TEA

Based on the functional characteristics of hMATE1 [6–8,14–19], the apical pH was suggested to be a crucial factor for its

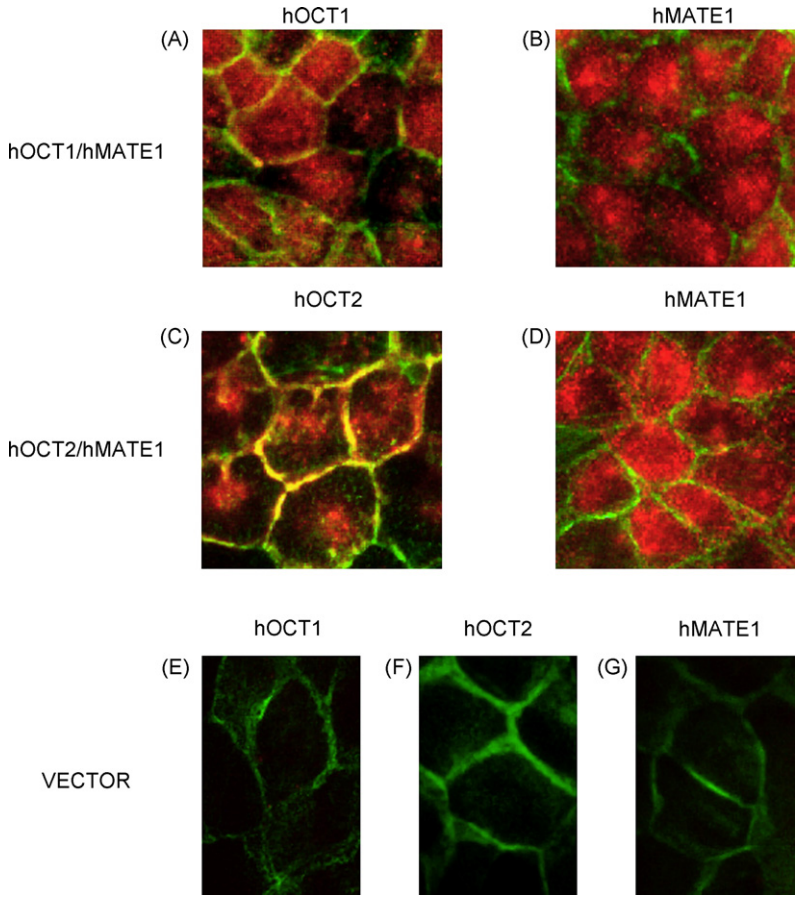

Fig. 1 – Immunofluorescence localization of hOCT and hMATE1 in the double transfected MDCK cells by immunofluorescence microscopy. MDCK-hOCT1/hMATE1 (A and B), MDCK-hOCT2/hMATE1 (C and D) and MDCK-vector (E–G) cells were stained with polyclonal antibody against hOCT1, hOCT2 or hMATE1 (red) and phalloidin (green). The yellow signals, which consist of hOCT1 or hOCT2 (red) and F-actin (green), were concentrated in basolateral side of double transfected MDCK cells (A and C). The hMATE1 expressions in double-transfected MDCK cells result in typical apical staining (B and D). No positive stainings for hOCT1, hOCT2 and hMATE1 (red) were observed in MDCK-vector cells (E–G).

transport characteristics. However, intracellular accumulation of [^{14}C]TEA was rapidly equilibrated by incubation time in MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cell monolayers (Fig. 3E and F). Because of these technical limitations, we examined the effect of the apical pH on the transcellular transport and accumulation of [^{14}C]TEA at 5 min with 4.7-cm^2 chamber.

As shown in Fig. 4, the basolateral-to-apical transport of [^{14}C]TEA was maximal at pH 6.5 (basolateral side: pH 7.4) in MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cell monolayers, and gradually decreased with change of the apical pH. Corresponding to the transport activity, the accumulation was increased by the alkalization of apical pH.

3.4. *Inhibitory effects of MPP and levofloxacin on the transcellular transport and cellular accumulation of TEA by MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cells*

In MDCK-hOCT1/hMATE1 cell monolayers, the basolateral-to-apical transport and cellular accumulation of [^{14}C]TEA was decreased in the presence of 10 mM MPP or 1 mM levofloxacin at the basolateral side with 4.7-cm^2 chamber (Fig. 5B and E). In MDCK-hOCT2/hMATE1 cell monolayers, MPP and levofloxacin decreased the transcellular transport of [^{14}C]TEA (Fig. 5C). Although the presence of MPP decreased the cellular accumulation of [^{14}C]TEA, the presence of levofloxacin significantly increased the cellular accumulation of [^{14}C]TEA (Fig. 5F).

3.5. *Inhibitory effect of levofloxacin on TEA uptake by HEK293 cells expressing hOCT1, hOCT2 or hMATE1*

Fig. 6 shows the inhibitory effect of levofloxacin on [^{14}C]TEA uptake by HEK293 cells transiently expressing hOCT1, hOCT2 or hMATE1. In the HEK293 cells expressing hOCT1 or hMATE1, the presence of levofloxacin markedly reduced the uptake of [^{14}C]TEA. In contrast, the [^{14}C]TEA uptake in the hOCT2 expressing cells was not changed with or without levofloxacin.

3.6. *Uptake of various cationic compounds in HEK293 cells transfected with hOCT1, hOCT2 or hMATE1*

Prior to experiment with double transfectants, the uptake of various organic cations by hOCT1, hOCT2 or hMATE1 was examined. The uptake of [^{14}C]TEA, [^{3}H]MPP, [^{14}C]metformin

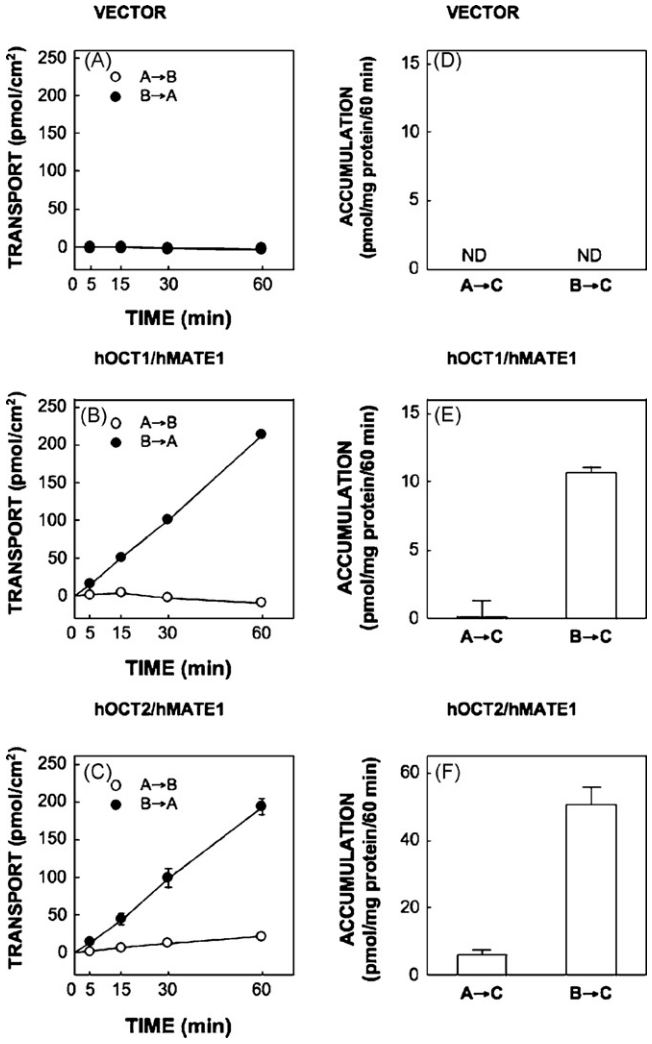

Fig. 2 – Transcellular transport (A–C) and cellular accumulation (D–F) of [^{14}C]TEA in MDCK-vector (A and D), MDCK-hOCT1/hMATE1 (B and E) and MDCK-hOCT2/hMATE1 (C and F) cell monolayers in 4.7-cm^2 chamber. The cells were incubated in medium containing 5 μM [^{14}C]TEA added to the basolateral (closed circle) or apical (open circle) side. The radioactivity on the opposite side was periodically measured. After a 60-min incubation, the radioactivity of solubilized cells was measured. Each point or column represents the mean ± S.E. for three monolayers from a typical experiment. N.D., not detected.

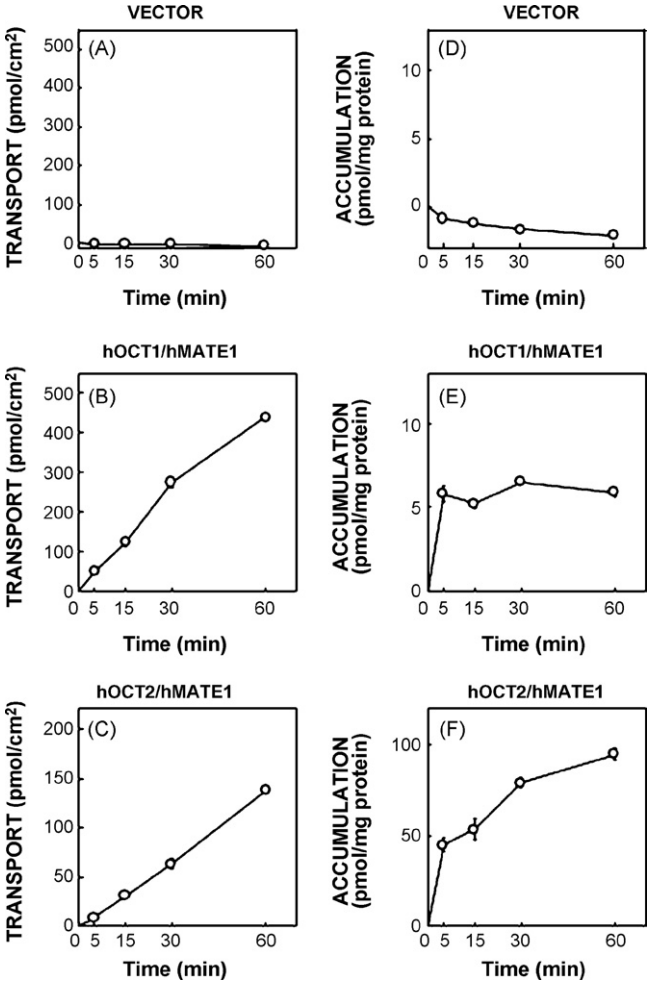

Fig. 3 – Time course of [^{14}C]TEA accumulation by MDCK-vector (A and D), MDCK-hOCT1/hMATE1 (B and E) and MDCK-hOCT2/hMATE1 (C and F) cell monolayers in 1.0-cm^2 chamber. The cells were incubated in medium containing 5 μM TEA added to the basolateral side. The radioactivity in the apical medium and solubilized cells were measured at the end of incubation. Each point represents the mean ± S.E. for three monolayers from a typical experiment.

and [^{3}H]cimetidine was markedly stimulated in hOCT1- and hOCT2-expressing cells (Table 1). The uptake of [^{14}C]creatinine and [^{14}C]guanidine was significantly increased in hOCT2-expressing cells, but not in hOCT1-expressing cells. [^{14}C]Procainamide and [^{3}H]quinidine were transported by hOCT1. Although [^{14}C]procainamide was also recognized, [^{14}C]quinidine was slightly, but not significantly, transported by hOCT2. hMATE1 extensively transported [^{14}C]TEA, [^{3}H]MPP, [^{14}C]metformin, [^{3}H]cimetidine, [^{14}C]creatinine and [^{14}C]procainamide after the pre-treatment with ammonium chloride (Table 2). The cellular accumulation of [^{14}C]guanidine and [^{3}H]quinidine was weakly but significantly increased in hMATE1-expressing cells compared to control cells.

3.7. *Transcellular transport and cellular accumulation of various cationic compounds*

Because the sum of the basolateral uptake and apical secretion of organic cations was supposed to reflect the in vivo biliary and tubular secretion, we examined the transcellular transport of these compounds using the established double transfectants with 1.0-cm^2 chamber (Tables 3 and 4). The basolateral-to-apical transport of [^{14}C]TEA, [^{3}H]MPP, [^{14}C]metformin, [^{3}H]cimetidine, [^{14}C]procainamide and [^{3}H]quinidine was much greater than the apical-to-basolateral transport by MDCK-hOCT1/hMATE1

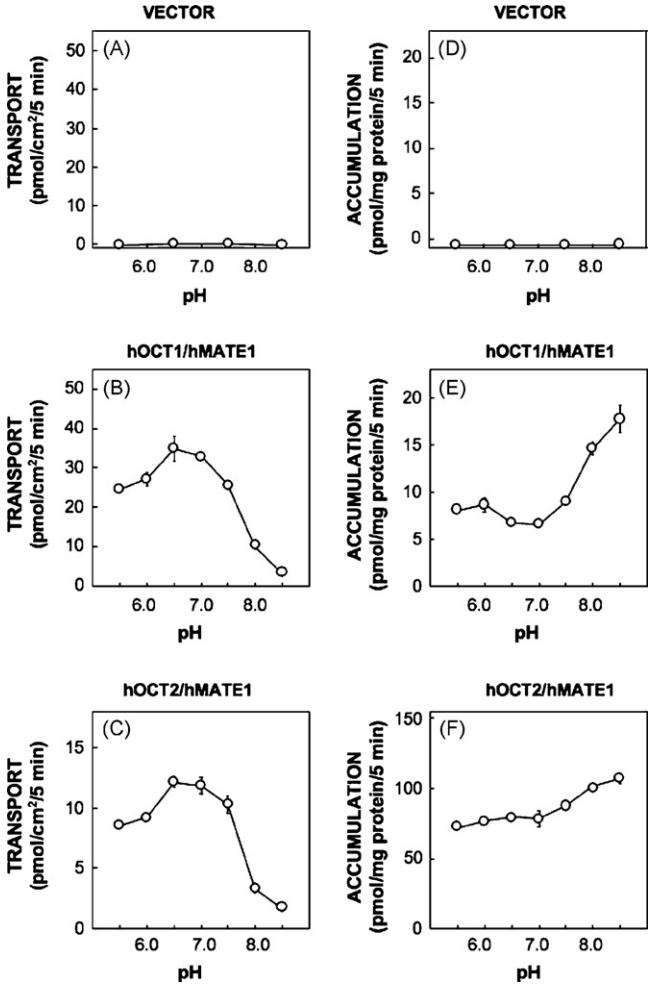

Fig. 4 – Effect of apical pH on the transcellular transport (A–C) and cellular accumulation (D–F) of [^{14}C]TEA in MDCK-vector (A and D), MDCK-hOCT1/hMATE1 (B and E), and MDCK-hOCT2/hMATE1 (C and F) cell monolayers in 4.7-cm^2 chamber. The cells were incubated in medium containing 5 μM [^{14}C]TEA added to the basolateral side for 5 min. The pH of the apical medium was between 5.5 and 8.5, and that of the basolateral medium was 7.4. The radioactivity in the apical medium and solubilized cells were measured at the end of incubation. Each point represents the mean ± S.E. for three monolayers from a typical experiment.

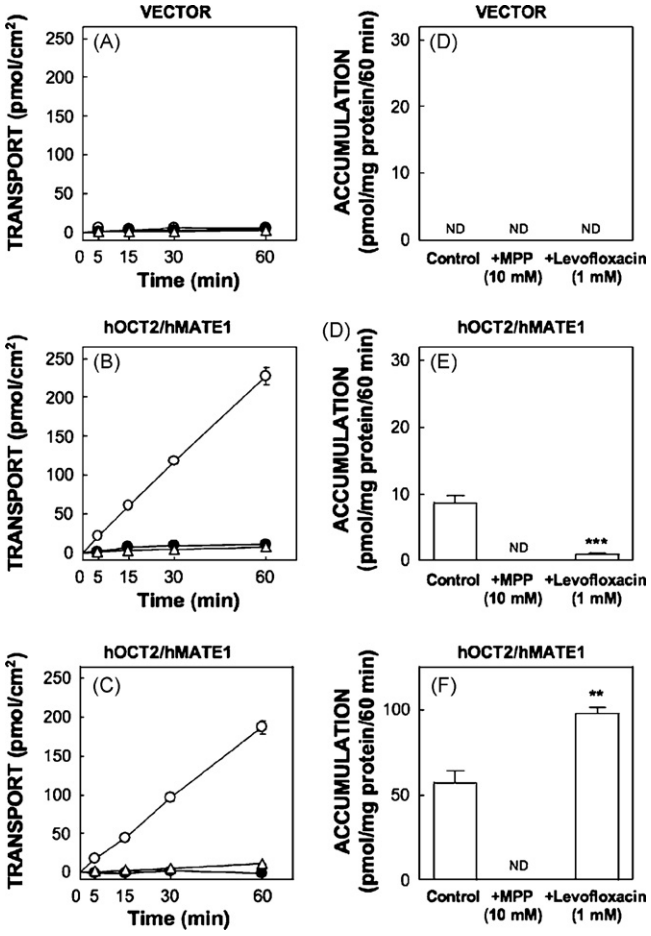

Fig. 5 – Effect of MPP and levofloxacin on the transcellular transport (A–C) and cellular accumulation (D–F) of [^{14}C]TEA in MDCK-vector (A and D), MDCK-hOCT1/hMATE1 (B and E), and MDCK-hOCT2/hMATE1 (C and F) cell monolayers in 4.7-cm^2 chamber. These cell monolayers were incubated in medium containing 5 μM [^{14}C]TEA added to the basolateral side in the absence (control, open circle) or presence of 10 mM MPP (+MPP, closed circle) or 1 mM levofloxacin (+levofloxacin, open triangle). The pH of the basolateral and apical incubation medium was 7.4 and 6.0, respectively. The radioactivity on the apical side was periodically measured. After a 60-min incubation, the radioactivity of solubilized cells was measured. Each symbol and column represents the mean ± S.E. for three monolayers from a typical experiment. N.D., not detected. $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from control cells.

and MDCK-hOCT2/hMATE1 cell monolayers (Tables 3 and 4). In the MDCK-hOCT1/hMATE1 cell monolayers, the cellular accumulation of these compounds was greater from the basolateral side than apical side (Table 3). Although the transcellular transport of [^{14}C]creatinine and [^{14}C]guanidine was slightly increased in MDCK-hOCT1/hMATE1 cell monolayers, there was no difference between apical-to-basolateral and basolateral-to-apical transport in the MDCK-hOCT2/hMATE1 cell monolayers. On the other hand, the cellular accumulation was also greater from the basolateral than apical side (Tables 3 and 4).

4. Discussion

The functional characterization of MATE1 and MATE2-K as the apical H^+/organic cation antiporter has been examined using transfected cells and isolated membrane vesicles [7,8,14–16,18,19]. However, most of the characteristics of MATE transporters were determined in the direction of uptake using an oppositely directed H^+ gradient. Therefore, examination of the H^+/organic cation antiport functions of MATE transporters

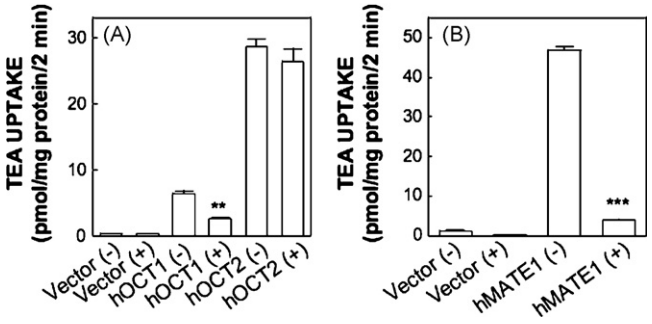

Fig. 6 – Effect of levofloxacin on the uptake of [^{14}C]TEA by HEK293 cells transiently expressing hOCT1, hOCT2 (A) and hMATE1 (B). The cells were incubated with medium containing 5 μM [^{14}C]TEA in the absence (−) or presence (+) of 1 mM levofloxacin at pH 7.4 for 2 min. For intracellular acidification, hMATE1 expressing cells were preincubated in medium containing 30 mM ammonium chloride for 20 min (B). The amount of substrate in the cells was determined by measuring the radioactivity of solubilized cells. Each column represents the mean ± S.E. for three monolayers. $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from the uptake of the absence of levofloxacin. .

in the direction of efflux has been considered to be difficult. The extracellular H^+ concentration-dependent efflux of preloaded TEA was showed by the CHO cells expressing rabbit (rb) MATE1 and rbMATE2-K [17]. However, it may be difficult to examine the efflux activity with lipophilic compounds in this method. We hypothesized that the ''efflux-function'' via MATE transporters can be revealed in polarized epithelial cells with the basolateral OCT system regardless of the lipophilicity of compounds. In the present study, we have successfully established double-transfected MDCK cells expressing both hOCT1 and hMATE1 or both hOCT2 and hMATE1 as an in vitro model of the organic cation transport system in the human hepatocyte and human tubular epithelial cell, respectively.

Typical and hydrophilic substrates for organic cation transporters, [^{14}C]TEA, [^{3}H]MPP, [^{14}C]metformin and [^{3}H]cimetidine were strongly transported by the HEK293 cells expressing either hOCT1, hOCT2 or hMATE1 (Tables 1 and 2). Indeed, metformin and cimetidine were indicated to be eliminated by renal tubular secretion [20–22], and the molecular mechanisms of renal secretion were revealed to be mediated by organic cation transport systems [8,23]. In the MDCK-hOCT2/hMATE1 cell monolayers, the unidirectional transcellular transport of these compounds from the basolateral to apical

Table 1 – Uptake of cationic compounds by HEK293 cells expressing hOCT1 or hOCT2

Cationic compounds	Vector (μl/mg protein/2 min)	hOCT1 (μl/mg protein/2 min)	hOCT2 (μl/mg protein/2 min)
[^{14}C]TEA	0.08 ± 0.00	2.64 ± 0.03**	2.86 ± 0.09**
[^{3}H]MPP	1.01 ± 0.01	8.72 ± 0.47**	7.17 ± 0.51**
[^{14}C]Metformin	0.18 ± 0.01	0.72 ± 0.04**	4.01 ± 0.08**
[^{3}H]Cimetidine	0.49 ± 0.02	0.86 ± 0.01**	1.31 ± 0.02**
[^{14}C]Creatinine	0.98 ± 0.01	0.93 ± 0.05	2.27 ± 0.09**
[^{14}C]Guanidine	2.23 ± 0.06	2.11 ± 0.07	5.71 ± 0.19**
[^{14}C]Procainamide	3.36 ± 0.18	5.13 ± 0.09**	5.32 ± 0.28**
[^{3}H]Quinidine	37.4 ± 0.43	47.8 ± 3.43*	43.4 ± 1.12

HEK293 cells were transfected with the empty vector, hOCT1 cDNA or hOCT2 cDNA. The cells were incubated with medium containing 5 μM [^{14}C]TEA 15 nM [^{3}H]MPP, 10 μM [^{14}C]metformin, 92 nM [^{3}H]cimetidine, 5 μM [^{14}C]creatinine, 5 μM [^{14}C]guanidine, 5 μM [^{14}C]procainamide and 56 nM [^{3}H]quinidine at pH 7.4 for 2 min. The amount of substrate in the cells was determined by measuring the radioactivity of solubilized cells. Date represents the mean ± S.E. for three monolayers. TEA, tetraethylammonium; MPP, 1-methyl-4-phenylpyridinium acetate.

* $P < 0.05$.

** $P < 0.01$ significantly different from vector-transfected cells.

Table 2 – Uptake of cationic compounds by HEK293 cells expressing hMATE1

Cationic compounds	Vector (μl/mg protein/2 min)	hMATE1 (μl/mg protein/2 min)
[^{14}C]TEA	0.09 ± 0.00	15.9 ± 0.34***
[^{3}H]MPP	0.85 ± 0.03	40.3 ± 1.36***
[^{14}C]Metformin	0.18 ± 0.01	24.9 ± 0.77***
[^{3}H]Cimetidine	0.53 ± 0.00	9.27 ± 0.38***
[^{14}C]Creatinine	0.41 ± 0.02	2.41 ± 0.11***
[^{14}C]Guanidine	1.36 ± 0.03	2.77 ± 0.27**
[^{14}C]Procainamide	6.78 ± 0.29	18.8 ± 0.41***
[^{3}H]Quinidine	38.9 ± 0.49	46.2 ± 0.37***

HEK293 cells transfected with the empty vector or hMATE1 cDNA. The cells were preincubated in medium containing 30 mM ammonium chloride for 20 min to make an intracellular acidification, and then incubated in medium containing radiolabeled compounds as described in the legend for Table 1. Data represents the mean ± S.E. for three monolayers.

** $P < 0.01$.

*** $P < 0.001$ significantly different from vector-transfected cells.

Table 3 – Transcellular transport and cellular accumulation of various cationic compounds in MDCK-hOCT1/hMATE1 cell monolayers

Cationic compounds	Transport (μl/cm²/30 min)		Accumulation (μl/mg protein/30 min)	
	A → B	B → A	A → C	B → C
[¹⁴C]TEA	N.D.	23.7 ± 0.64***	N.D.	1.92 ± 0.07**
[³H]MPP	5.40 ± 0.24	117 ± 2.58***	0.49 ± 0.02	2.40 ± 0.04***
[¹⁴C]Metformin	2.17 ± 0.30	31.3 ± 0.59***	0.11 ± 0.04	2.13 ± 0.19***
[³H]Cimetidine	1.67 ± 0.61	49.4 ± 1.06***	0.79 ± 0.04	1.51 ± 0.10**
[¹⁴C]Creatinine	1.94 ± 0.27	3.19 ± 0.11*	N.D.	0.83 ± 0.08**
[¹⁴C]Guanidine	14.5 ± 0.33	22.5 ± 0.35***	0.11 ± 0.08	2.49 ± 0.27***
[¹⁴C]Procainamide	N.D.	27.4 ± 2.09**	0.19 ± 0.04	1.12 ± 0.06***
[³H]Quinidine	0.48 ± 0.30	95.0 ± 1.07***	18.2 ± 0.32	51.3 ± 2.61***

The cells were incubated in medium containing radiolabeled compounds added to the basolateral (B → A) or apical (A → B) side as described in the legend for Table 1. The radioactivity in the opposite side and solubilized cells (C) was measured at the end of incubation. Data represents the mean ± S.E. for three monolayers from typical experiments. N.D., not detected.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$ significantly different from apical to basolateral (transport) or apical to cell (accumulation).

Table 4 – Transcellular transport and cellular accumulation of various cationic compounds in MDCK-hOCT2/hMATE1 cell monolayers

Cationic compounds	Transport (μl/cm²/30 min)		Accumulation (μl/mg protein/30 min)	
	A → B	B → A	A → C	B → C
[¹⁴C]TEA	0.63 ± 0.34	19.8 ± 0.57***	0.33 ± 0.27	14.0 ± 0.36***
[³H]MPP	10.3 ± 0.33	120 ± 2.20***	1.71 ± 0.03	11.8 ± 0.72***
[¹⁴C]Metformin	0.23 ± 0.07	15.0 ± 0.60***	0.04 ± 0.04	13.6 ± 0.28***
[³H]Cimetidine	N.D.	17.9 ± 0.48***	0.22 ± 0.04	5.85 ± 0.28***
[¹⁴C]Creatinine	1.84 ± 0.20	1.80 ± 0.07	N.D.	1.43 ± 0.09**
[¹⁴C]Guanidine	14.2 ± 3.14	18.7 ± 0.10	1.63 ± 0.43	11.2 ± 0.31***
[¹⁴C]Procainamide	N.D.	9.06 ± 0.27***	0.15 ± 0.04	4.30 ± 0.23***
[³H]Quinidine	1.23 ± 0.21	72.9 ± 5.27***	18.5 ± 0.55	77.8 ± 4.31***

The cells were incubated in medium containing radiolabeled compounds added to the basolateral (B → A) or apical (A → B) side as described in the legend for Table 1. The radioactivity in the opposite side and solubilized cells (C) was measured at the end of incubation. Data represents the mean ± S.E. for three monolayers from typical experiments. N.D., not detected.

** $P < 0.01$.

*** $P < 0.001$ significantly different from apical to basolateral (transport) or apical to cell (accumulation).

side was observed, corresponding to the tubular secretion (Table 4). These results suggested that MDCK-hOCT2/hMATE1 cell monolayers successfully reproduced the renal tubular secretion, and this transfectant can be used to examine the tubular secretion via the human organic cation transport system. The MDCK-hOCT1/hMATE1 cell monolayers also showed the unidirectional transcellular transport of [¹⁴C]TEA, [³H]MPP, [¹⁴C]metformin and [³H]cimetidine from the basolateral side, corresponding to biliary excretion (Table 3). Considering the in vivo pharmacokinetics of cimetidine and metformin [21,22], the present results suggest that intestinal reabsorption of these drugs after biliary secretion may have occurred. Although the report that MDCK cells originally expressed an OCT on basolateral side [24], MDCK-vector cell monolayers in the present study did not show the transcellular transport and accumulation of [¹⁴C]TEA (Fig. 2A and D). In addition, we previously examined that the radioactivity of [¹⁴C]TEA added to the basolateral side was not transported to the apical side, in the MDCK cells stably expressing rat OCT1 and OCT2 (data not shown) [25], and then, the function corresponding to hMATE1 was indicated to be lacked in MDCK cells. Therefore, we consider that MDCK cells in the present study were not expressing an original OCT on basolateral side.

Immunostaining against hOCT1 or hOCT2 in double transfected MDCK cells was observed as yellow signals merged with green phalloidin and red antibodies (Fig. 1A and C). Despite the red staining was observed at the intracellular space of MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cells, they were considered to be non-specific signals from the functional analysis. Therefore, we confirmed that hOCT1 and hOCT2 were localized on the basolateral side, and hMATE1 on the apical side in double-transfected MDCK cells.

The uptake experiments using HEK293 cells expressing hOCT1, hOCT2 or hMATE1 gave results that contradicted the in vivo data in the cases of procainamide and quinidine, the lipophilic cations. In the human pharmacokinetic analysis, the renal clearance of procainamide was decreased by the coadministration of cimetidine [26] and the renal clearance of unbound quinidine was greater than the clearance of creatinine [27]. These reports suggested that procainamide

and quinidine undergo active tubular secretion in the human kidney. In uptake experiments, the accumulation of [^{14}C]procainamide and [^{3}H]quinidine by HEK293 cells expressing hOCT1, hOCT2 or hMATE1 was not so great compared to that by vector-transfected control cells, unlike in the case of [^{14}C]metformin and [^{3}H]cimetidine (Tables 1 and 2). These results could not clearly indicate that the renal secretion of procainamide and quinidine was mediated by the organic cation transport system. In the transcellular transport experiments with MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cell monolayers, clear directional transport of [^{14}C]procainamide and [^{3}H]quinidine was observed from the basolateral to apical side, corresponding to biliary excretion and renal tubular secretion (Tables 3 and 4). These results suggested that the biliary excretion and renal tubular secretion of procainamide and quinidine are involved in the basolateral uptake by hOCT and apical efflux by hMATE1 in humans. Because uptake experiments can not discriminate between the specific intracellular accumulation by transporters and non-specific binding to the cell surface and/or diffusion dependent on lipophilicity, it was considered that such experiments alone could not clarify the tubular secretion of procainamide and quinidine via the organic cation transport system. The double-transfected MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cells clearly solved these technical limitations of the uptake experiments, and would be useful in vitro tools to examine the biliary and renal tubular secretion of cationic drugs in humans.

In the liver, the existence of the H^+/organic cation antiporter has been examined using canalicular membrane vesicles [28]. In this report, an outwardly directed H^+ gradient stimulated the TEA uptake by canalicular membrane vesicles, suggesting that organic cation transport across the hepatic canalicular membranes is mediated by the H^+/organic cation antiporter. Consistent with this report, hMATE1 is expressed in the liver as well as kidney [6]. In contrast, the source of the H^+ gradient is considered to differ between the kidney and the liver. In the brush-border membranes of renal proximal tubules, the Na^+/H^+ antiporter NHE3 and H^+-ATPase are considered to produce an inward H^+ gradient [29,30]. However, the Na^+/H^+ exchanger is not functional in the canalicular membranes in the liver [31,32]. It remains unclear whether hMATE1 functions well in the liver like in the kidney. A comparison between the in vivo hepatic pharmacokinetics and the transcellular transport by the MDCK-hOCT1/hMATE1 cells may be revealed the physiological significance of hepatic hMATE1 as a detoxicating molecule.

Previously, we found that the new-quinolone antibiotics ofloxacin and levofloxacin strongly inhibited the transport of TEA via the H^+/organic cation antiport system using the isolated rat renal brush-border membrane vesicles and the LLC-PK_1 cell monolayers [33,34]. Moreover, we have reported that levofloxacin inhibited the transport of [^{14}C]TEA by hMATE1 [8], but not by hOCT2 [35]. However, the effect of levofloxacin on the renal handling of cationic drugs in humans has not been elucidated. In the MDCK-hOCT2/hMATE1 cell monolayers, the transcellular transport of [^{14}C]TEA was markedly decreased in the presence of 10 mM MPP and 1 mM levofloxacin in the basolateral chamber (Fig. 5C). On the other hand, the cellular accumulation of [^{14}C]TEA was decreased by MPP and increased by levofloxacin (Fig. 5F). In MDCK-hOCT1/hMATE1 cell monolayers, the transcellular transport and accumulation of [^{14}C]TEA was inhibited by 10 mM MPP and 1 mM levofloxacin (Fig. 5B and E). Considering the results by the single transfectants of hOCT1, hOCT2 and hMATE1 (Fig. 6), these results in the MDCK-hOCT2/hMATE1 monolayers suggested that levofloxacin selectively inhibited the transport of [^{14}C]TEA by apical hMATE1 rather than basolateral hOCT2, and therefore, coadministration of levofloxacin may increase the tubular accumulation of cationic drugs and the risk of subsequent drug-induced nephrotoxicity.

hMATE2-K as well as hMATE1 was found in the human kidney, and both transporters were suggested to play important roles in the urinary secretion of cationic compounds [7]. Although these transporters have a similar substrate specificity, some differences were found. The amino beta-lactam antibiotic cephalexin was preferentially transported by hMATE1, and the anticancer agent oxaliplatin was by hMATE2-K [8,15]. However, the substrate specificity was mainly examined with uptake experiments. Because hMATE1 and hMATE2-K act as efflux transporters, the construction of MDCK cells expressing basolateral hOCT2 and apical hMATE2-K will be required in future. A comparison of substrate specificity between hMATE1 and hMATE2-K for basolateral-to-apical transport using double transfectants would clearly distinguish the physiological characteristics of these two transporters.

In conclusion, we have successfully established double-transfected MDCK cells expressing both hOCT1 and hMATE1 or both hOCT2 and hMATE1. These double transfectants represented the vectorial transcellular transport of cationic drugs elucidating at least a part of the molecular mechanisms of hepatic- and/or renal-handling of cationic drugs in humans. The MDCK-hOCT1/hMATE1 and MDCK-hOCT2/hMATE1 cells are suggested to be useful for determining the routes by which newly synthesized cationic compounds are eliminated, or predicting transporter-mediated drug interaction in humans.

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