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Mechanisms for membrane transport of metformin in human intestinal epithelial Caco-2 cells

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ABSTRACT: The aim of the present study was to investigate the mechanisms for membrane transport of metformin in human intestinal epithelial Caco-2 cells. The mRNA of not only organic cation transporter (OCT) 3, but also OCT1 and OCT2, was expressed in Caco-2 cells. The uptake of 100 μM metformin at the apical membrane of Caco-2 cells grown on porous filter membrane was significantly greater than that at the basolateral membrane. The apical uptake of 100 μM metformin in Caco-2 cells grown on plastic dishes was inhibited significantly by 1 mM unlabeled metformin, quinidine and pyrilamine, indicating that a specific transport system is involved in the apical uptake of metformin in Caco-2 cells. The apical uptake of 100 μM metformin in Caco-2 cells was decreased by acidification of the medium, but not increased by alkalization. In addition, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (a protonophore) had no effect on the apical uptake of metformin in Caco-2 cells at apical medium pH 8.4. These findings suggested that the apical uptake of metformin in Caco-2 cells is mediated at least partly by OCTs, but that the postulated H^+/tertiary amine antiport system is not responsible for the apical uptake of metformin. Copyright © 2011 John Wiley & Sons, Ltd.

Key words: metformin; organic cation transporter; Caco-2 cell

Introduction

Metformin, a hydrophilic biguanide compound, is an oral antihyperglycemic agent that has been used widely in the treatment of type 2 diabetes mellitus [1]. It lowers the blood glucose concentration by decreasing hepatic glucose production and intestinal glucose absorption, and by increasing glucose uptake into skeletal muscle and adipose tissue [1,2]. Compared with another biguanide agent, phenformin, metformin has a low incidence of adverse reactions, such as lactic acidosis [3]. Orally administered metformin is slowly absorbed from the proximal small intestine, and the unchanged drug is predominantly excreted in the urine [2,4]. The oral bioavailability of metformin ranges between 40% and 60%, and is inversely correlated with the dose ingested, suggesting that metformin is absorbed by the specific and saturable transport system [4,5].

Metformin and another hydrophilic organic cation, tetraethylammonium (TEA), are well-known substrates of organic cation transporters (OCTs) [6–13]. Human (h) OCT1 and hOCT2 are mainly expressed in the sinusoidal membrane of the liver and in the basolateral membrane of the kidney, respectively, and are responsible for the hepatic uptake and renal excretion of cationic drugs [6]. Müller *et al.* evaluated the localization of OCTs using immunohistochemical staining in a segment of human jejunum that was surgically removed during the preparation of a small gastrointestinal anastomosis [14].

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hOCT1 immunolabeling was observed mainly in the cytoplasm and the lateral membrane of the enterocytes. In addition, no specific immunolabeling was observed with the antibody against hOCT2. On the other hand, hOCT3 is expressed mainly in the brush border membrane of the enterocytes [14].

It has been reported that the transport of tertiary amines across the intestinal epithelial cells is mediated by an unidentified H^+/tertiary amine antiport system [15–19]. We previously evaluated the membrane transport of quinidine in human intestinal epithelial Caco-2 cells grown on plastic dishes, and reported that the cellular uptake of quinidine in Caco-2 cells was temperature-dependent, and markedly increased by alkalization of the apical medium [15]. In addition, the 15 min uptake of quinidine in Caco-2 cells was inhibited by 5 mM hydrophobic organic cations, and the rank order of the inhibitory effects of tertiary amines was imipramine > quinidine > diphenhydramine [16]. These results suggested that an unidentified H^+/tertiary amine antiport system is involved in the influx of quinidine in Caco-2 cells [15,16]. In addition, Mizuuchi *et al.* investigated the mechanisms responsible for the transcellular transport of diphenhydramine in Caco-2 cells [18,19]. The uptake of diphenhydramine at the apical membrane in Caco-2 cells was pH- and temperature-dependent, and was inhibited by chlorpheniramine, procainamide and imipramine [18,19]. The authors thought that the H^+/tertiary amine antiport system specifically recognizes *N*-dimethyl or *N*-diethyl moieties in tertiary amine compounds [19]. However, it is still unclear whether the postulated H^+/tertiary amine antiport system is responsible for the intestinal absorption of metformin with an *N*-dimethyl moiety.

The present study investigated the mechanisms for membrane transport of metformin in Caco-2 cells. That is, the membrane transport characteristics of metformin in Caco-2 cells were compared with those of TEA and quinidine, which were utilized as positive controls for OCTs and the H^+/tertiary amine antiport system, respectively. In addition, TEA is not an inhibitor (substrate) of the postulated H^+/tertiary amine antiport system [15–17].

Materials and Methods

Materials

[^{14}C]Metformin hydrochloride (1.82 GBq/mmol) and [^{14}C]mannitol (1.96 GBq/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). [^{14}C]TEA bromide (2.04 GBq/mmol), [^{3}H]quinidine (740 GBq/mmol) and [^{3}H]mannitol (740 GBq/mmol) were purchased from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). Metformin hydrochloride, quinidine hydrochloride monohydrate and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) were purchased from Sigma Aldrich (St Louis, MO, USA). TEA chloride was obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of the highest purity available.

Cell culture

Caco-2 cells at passage 43 were obtained from the Riken Bioresource Center (Tsukuba, Japan). All experiments were carried out with Caco-2 cells between passages 59 and 70. The cells were maintained by serial passage in plastic dishes with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Biowest Inc., Nuaille, France) in an atmosphere of 5% CO_2–95% air at 37 °C.

Caco-2 cells were seeded at a density of 5×10^5 cells/cm^2 on a 0.9 cm^2 porous filter membrane (0.4 μm pore size) in a Falcon® multiwell™ plate (BD Bioscience, Franklin Lakes, NJ, USA) to evaluate the uptake of cationic drugs at the apical and basolateral membrane. The seeded cells were maintained for 21 days to prepare differentiated cell monolayers. The maturity of the monolayer was judged by transepithelial electrical resistance (TEER), which was measured using a Millicell-ERS resistance system (Millipore, Bedford, MA, USA). Caco-2 cell monolayers whose TEER was above 1000 Ω cm^2 were used to assess the uptake transport of cationic drugs. In order to evaluate the uptake of cationic drugs at the apical membrane and the mRNA expression of OCTs, Caco-2 cells were seeded at a density of 5×10^5 cells/cm^2 on a 3.8 cm^2 plastic dish using a Falcon® multiwell™ plate (BD Bioscience, Franklin Lakes, NJ, USA), and maintained for 21 days.

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Real-time PCR assay of the mRNA of multidrug resistance protein (MDR) 1 and OCTs

Total RNA was isolated from Caco-2 cells using an RNeasy® Mini Kit, QIAshredder, and RNase-Free DNase Set (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription of extracted total RNA was performed using an Omniscript® RT kit (QIAGEN) and random hexamer (QIAGEN) according to the manufacturer's instructions. PCR was carried out on the MX3000P® QPCR System (Stratagene, LaJolla, CA, USA) using SYBR® Premix Ex Taq™ (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Primer sequences for MDR1, OCT1, OCT2, OCT3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported elsewhere [20–22]. Cycling conditions were 1 cycle for 30 s at 95 °C, followed by 45 cycles of 5 s denaturation at 95 °C, 20 s annealing at 60 °C, and 15 s extension at 72 °C. The mRNA level of MDR1 and OCTs was normalized according to the GAPDH mRNA level, and the ratio was presented using a common logarithm.

Cellular uptake of TEA, quinidine and metformin in Caco-2 cell monolayers grown on a porous filter membrane

The cellular uptake of TEA, quinidine and metformin was examined using Caco-2 cells grown on a porous filter membrane. The composition of the incubation medium was as follows: 125 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 $MgSO_4 \cdot 7H_2O$, 25 mM 2-[4-(2-hydroxyethyl)-1-piperazyl]ethanesulfonic acid (HEPES) and 100 μM unlabeled drug (pH 7.4). In addition, 1 mM sodium pyruvate was added to the incubation medium in order to maintain the cell viability during 90 min drug uptake experiments. The volume of the incubation medium was 1 ml and 2.3 ml for inside (apical side) and outside (basolateral side) of the insert, respectively. The monolayers were first pre-incubated for 60 min at 37 °C with incubation medium to equilibrate the drug concentration. The incubation medium was replaced with incubation medium containing the radio-labeled drug (0.5–1.0 μCi/ml) on the apical side to examine the apical uptake of the drug. After the monolayers were incubated with the radio-labeled drug for another 90 min at 37 °C, they were immediately washed with ice-cold phosphate buffer and collected. Radioactivity in the cells was determined using a liquid scintillation counter, and normalized against the initially applied doses. [^{3}H]Mannitol was used to estimate the extracellular trapping of [^{14}C]metformin and [^{14}C]TEA, and [^{14}C]mannitol was used to estimate that of [^{3}H]quinidine. The uptake of the cationic drugs on the basolateral side was examined in the same manner.

Cellular uptake of TEA, quinidine and metformin in Caco-2 cells grown on plastic dishes

The cellular uptake of TEA, quinidine and metformin was examined using Caco-2 cells grown on plastic dishes of a multiwell plate. In order to evaluate the effect of extracellular pH on the cellular uptake of cationic drugs, HEPES (pH 6.5–7.5) in the incubation medium was replaced with 2-(*N*-morpholino)ethanesulfonic acid (pH 5.5) or Tris (pH 8.4–8.5). The cells were first pre-incubated for 60 min at 37 °C with 2 ml incubation medium to equilibrate the drug concentration. The incubation medium was replaced with 500 μl fresh incubation medium containing the radio-labeled drug (1.0 μCi/ml). After the cells were incubated with the radio-labeled drug for other 10–30 min at 37 °C, they were immediately washed with ice-cold phosphate buffer and collected. Radioactivity in the cells was determined as described above. Radio-labeled mannitol was used to estimate the extracellular trapping of cationic drugs.

The effect of various cationic drugs on the apical uptake of TEA, quinidine and metformin in Caco-2 cells grown on plastic dishes of a multiwell plate was evaluated at 37 °C. Briefly, the cells were first pre-incubated for 55 min with 2 ml incubation medium, followed by 5 min incubation with 500 μl fresh incubation medium supplemented with 1 mM various cationic drugs. The incubation medium was replaced with 500 μl incubation medium containing the radio-labeled drug (1.0 μCi/ml) and 1 mM various cationic drugs. After the cells were incubated with the radio-labeled drug for other 30 min at 37 °C, they were immediately

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washed with ice-cold phosphate buffer and collected. This study also evaluated the effect of FCCP, a protonophore, on the uptake of TEA, quinidine and metformin in Caco-2 cells grown on plastic dishes at 37 °C. That is, the cells were first pre-incubated for 40 min with 2 ml incubation medium (pH 7.4 or 8.4), followed by 20 min incubation with 500 µl fresh incubation medium supplemented with 50 µM FCCP [23,24]. The incubation medium was replaced with 500 µl incubation medium containing the radio-labeled drug (1.0 µCi/ml) and 50 µM FCCP. After the cells were incubated with the radio-labeled drug for another 10–20 min at 37 °C, they were immediately washed with ice-cold phosphate buffer and collected. Radioactivity in the cells was determined as described above. Radio-labeled mannitol was used to estimate the extracellular trapping of cationic drugs.

Statistical analysis

Values are expressed as the mean ± SE. The statistical significance of differences between two groups was tested using Student's *t*-test, provided that the variances of the groups were similar. If this was not the case, the Mann-Whitney *U*-test was applied. Multiple comparisons were performed using Scheffé's test following one-way ANOVA, provided that the variances of groups were similar. If this was not the case, a Scheffé-type test was applied following Kruskal-Wallis analysis. A p value less than 0.05 was considered to be statistically significant.

Results

Expression of mRNA of MDR1 and OCTs in Caco-2 cells

Metformin and TEA are well-known substrates for OCT1, OCT2 and OCT3 [6–13]. Firstly, the mRNA expression level of these OCTs in Caco-2 cells was evaluated (Figure 1). In addition, the mRNA expression of MDR1 was evaluated as a control. The mRNA expression of OCT3 in Caco-2 cells was higher than that of OCT1 and OCT2 (Figure 1). However, the mRNA expression of OCT3 was about 10% of that of MDR1.

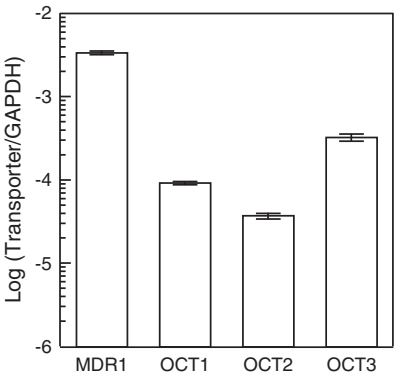

Figure 1. Expression of mRNA of MDR1 and OCTs in Caco-2 cells. Each column represents the mean ± SE of 4–7 experiments

Cellular uptake of TEA, quinidine and metformin in Caco-2 cell monolayers grown on a porous filter membrane

Next the cellular uptake of 100 µM metformin was compared with that of 100 µM TEA and quinidine in Caco-2 cell monolayers grown on a porous filter membrane (Figure 2). As shown in Figure 2A, the 90 min uptake of TEA at the apical membrane (mean: 97 pmol) was 12.3-fold greater than that at the basolateral membrane (mean: 7.9 pmol). The uptake of quinidine at the apical membrane (mean: 3437 pmol) was 7.0-fold higher than that at the basolateral membrane (mean: 492 pmol) (Figure 2B), which was consistent with our previous finding [15]. Similarly, the uptake of metformin at the apical membrane (mean: 300 pmol) was 14.3-fold greater than that at the basolateral membrane (mean: 21 pmol) (Figure 2C). In addition, the uptake rate of metformin for 90 min was faster than that of TEA, but was much slower than that of quinidine. That is, the uptake of TEA, quinidine and metformin at the apical membrane of Caco-2 cells was greater than that at the basolateral membrane, suggesting that the specific transport system is involved in the uptake of metformin, as well as TEA and quinidine, at the apical membrane of Caco-2 cells.

Transport characteristics of TEA, quinidine and metformin at the apical membrane in Caco-2 cells grown on plastic dishes

To investigate the mechanisms for transport of metformin at the apical membrane of Caco-2 cells,

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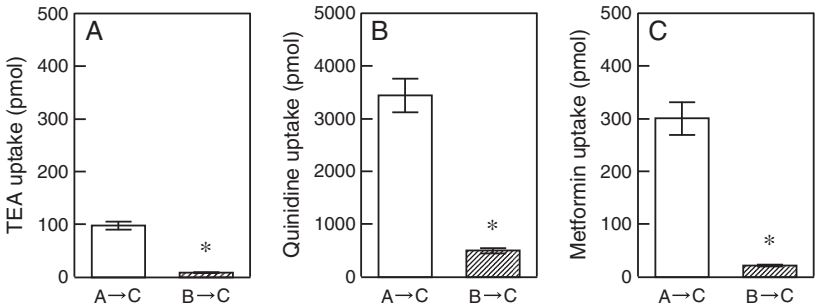

Figure 2. Cellular uptake of 100 μM TEA (A), quinidine (B) and metformin (C) at the apical and basolateral membrane in Caco-2 cells grown on a porous filter membrane. The cells were incubated with the radio-labeled drug for 90 min. Open and hatched columns represent the cellular accumulation from the apical side (A → C) and basolateral side (B → C), respectively. Each column represents the mean ± SE of 8–14 experiments. *$p < 0.05$, significantly different from (A → C)

the uptake characteristics of TEA, quinidine and metformin were evaluated in Caco-2 cells grown on plastic dishes. Figure 3 shows the 30 min uptake of TEA, quinidine and metformin in Caco-2 cells in the presence of 1 mM of various cationic drugs. The apical uptake of TEA was significantly inhibited by 1 mM hydrophilic and also hydrophobic cationic drugs, and the rank order of the inhibitory effects of cationic drugs on the uptake of TEA was pyrilamine ≈ quinidine > procainamide > cimetidine > metformin > unlabeled TEA (Figure 3A). On the other hand, the uptake of quinidine was not significantly inhibited by hydrophilic and also hydrophobic cations under the present 'long-uptake' and 'thin-inhibitor' conditions (Figure 3B) [16]. The apical uptake of metformin was inhibited by various cationic drugs, although the effect of TEA, procainamide and cimetidine was not statistically significant (Figure 3C). The rank order of the inhibitory effects of cationic drugs on the uptake of metformin was pyrilamine > quinidine > unlabeled metformin > cimetidine > procainamide > TEA (Figure 3C).

The transport activity of OCTs as well as the postulated H^+/tertiary amine antiport system is known to be pH-dependent [7–9]. Figure 4 shows the effect of extracellular pH on the apical uptake of TEA, quinidine and metformin in Caco-2 cells. The uptake of TEA, quinidine and metformin at the apical membrane in Caco-2 cells was decreased by acidification of the apical medium (Figure 4). The uptake of TEA and metformin was not increased at pH 8.5 compared with pH 7.5, whereas that of quinidine was significantly increased by alkalization of the apical medium (Figure 4).

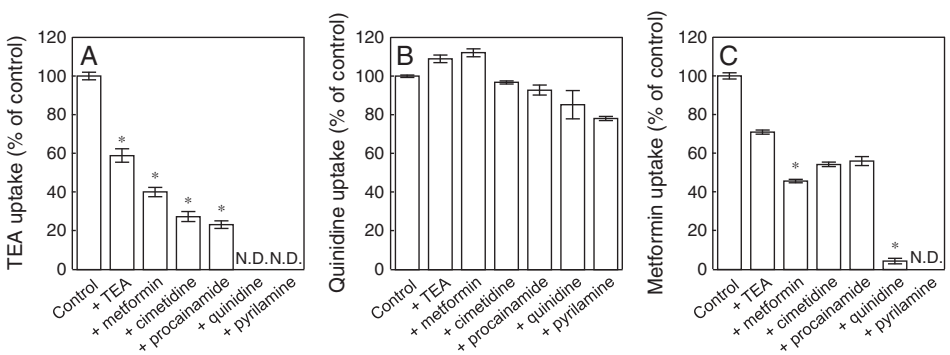

Figure 3. Effect of various compounds on the uptake of 100 μM TEA (A), quinidine (B) and metformin (C) in Caco-2 cells grown on plastic dishes. The cells were incubated with the radio-labeled drug for 30 min in the presence of 1 mM organic cations. Each column represents the mean ± SE of 5–15 experiments. N.D., not detected. *$p < 0.05$, significantly different from control

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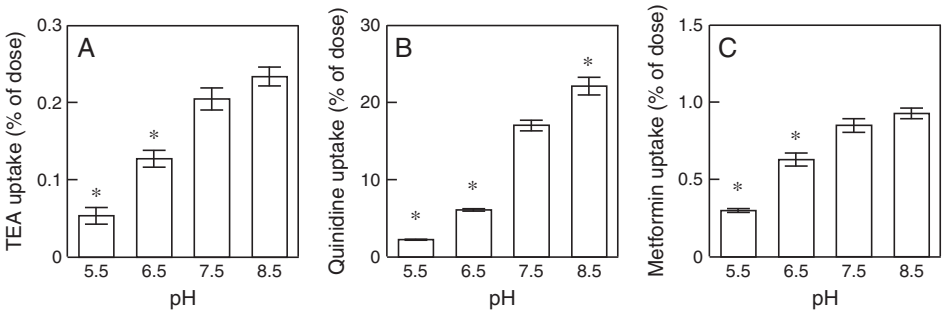

Figure 4. Effect of extracellular pH on the uptake of 100 μM TEA (A), quinidine (B) and metformin (C) in Caco-2 cells grown on plastic dishes. The cells were incubated with the radio-labeled drug for 30 min. Each column represents the mean ± SE of six experiments. *$p < 0.05$, significantly different from pH 7.5

The study further evaluated whether or not the apical uptake of metformin in Caco-2 cells is coupled with the outward H^+ gradient. Figure 5 shows the effect of 50 μM FCCP, which is known to dissipate transmembrane H^+ gradients [23,24], on the apical uptake of TEA, quinidine and metformin in Caco-2 cells. The apical uptake of TEA at apical medium pH 8.4 was virtually not altered by 50 μM FCCP (Figure 5A). On the other hand, the uptake of quinidine was significantly decreased by 50 μM FCCP at apical medium pH 8.4 (Figure 5B), confirming that the H^+/tertiary amine antiport system is involved in the uptake of quinidine at the apical membrane of Caco-2 cells [15,16]. However, FCCP had no significant effect on the uptake of metformin at apical medium pH 8.4 (Figure 5C). The present findings suggested that the apical uptake of metformin in Caco-2 cells is mediated at least partly by OCTs, but that the postulated H^+/tertiary amine antiport system is not responsible for the apical uptake of the drug.

Discussion

In the present study, the intestinal transport characteristics of metformin were compared with those of TEA and quinidine using Caco-2 cells. The mRNA of not only OCT3, but also OCT1 and OCT2, was expressed in Caco-2 cells. The apical uptake of metformin as well as TEA in Caco-2 cells seemed to be mediated at least partly by OCTs. In addition, the transport characteristics of metformin in Caco-2 cells were found to be different from those of quinidine.

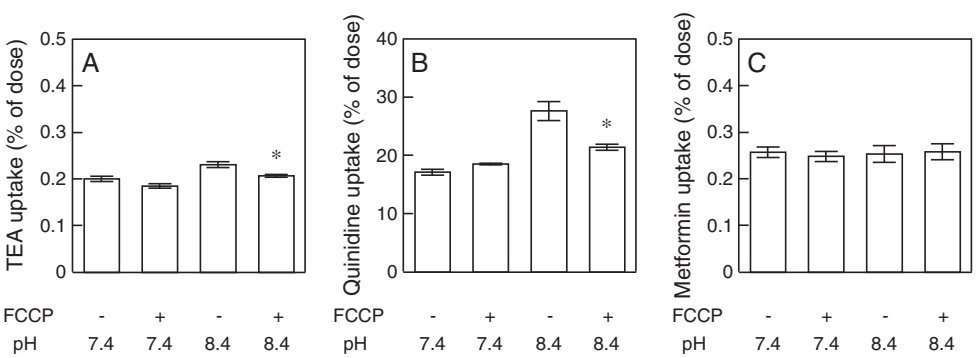

Figure 5. Effect of FCCP on the uptake of 100 μM TEA (A), quinidine (B) and metformin (C) in Caco-2 cells grown on plastic dishes. The cells were incubated with [^{14}C]TEA, [^{3}H]quinidine and [^{14}C]metformin for 20, 10 and 10 min, respectively, in the absence and presence of 50 μM FCCP. Each column represents the mean ± SE of 6–9 experiments. *$p < 0.05$, significantly different from FCCP(−)

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Müller *et al.* evaluated the protein expression and intracellular localization of hOCTs using immunohistochemical staining in Caco-2 cells [14]. The immunolabeling with the antibody against hOCT1 was only very weak. On the other hand, weak but significant labeling was observed with the antibody against hOCT2, and strong labeling was obtained with the antibody against hOCT3. Immunoreactivity for hOCT2 and hOCT3 in Caco-2 cells was detected within the cytoplasm and at the cell borders, suggesting that hOCT2 and hOCT3 are present within the plasma membrane and/or in vesicular compartments below the plasma membrane [14]. However, it is still unclear whether hOCT2 and hOCT3 are localized on the apical membrane of Caco-2 cells.

The transport of organic cations by OCTs is electrogenic and facilitated by the inside-negative membrane potential, but OCTs do not use the directed H^+ gradient as the driving force [6]. Substrate and inhibitor specificities of OCT1, OCT2 and OCT3 overlap broadly; however, individual OCTs have different affinities of the same substrate and inhibitor [6]. Indeed, the affinity of OCTs for metformin seems to be different from that for TEA. Kimura *et al.* performed the kinetic evaluation of metformin transport in hOCT1- and hOCT2-expressing human embryonic kidney 293 (HEK293) cells, and reported that hOCT1 and hOCT2 transported metformin with apparent K_m values of 1.47 mM and 0.99 mM, respectively [11]. Recently, Nies *et al.* have evaluated the transport activity of metformin in hOCT1- and hOCT3-expression Chinese hamster ovary (CHO) cells, and reported that the apparent K_m values of metformin for hOCT1 and hOCT3 were 2.16 mM and 2.26 mM, respectively [12]. On the other hand, the K_m values of OCTs for TEA seemed to be lower than those for metformin. That is, it was reported that the K_m value of hOCT1, hOCT2 and hOCT3 for TEA was 229 μM, 76 μM and 921 μM, respectively [6,13]. In the present study, the apical uptake of TEA and metformin was inhibited by cationic drugs, but the rank order of inhibitory effects of cationic drugs on the uptake of metformin was not consistent with that of TEA (Figure 3A, 3C). One possible explanation for the finding is that the contribution of individual OCTs to the uptake of metformin may be different from that of TEA. Further studies will need to be performed to clarify which OCT is mainly responsible for the absorption of metformin in the human intestine.

In conclusion, the uptake of metformin at the apical membrane in Caco-2 cells may be mediated at least partly by OCTs. However, the postulated H^+/tertiary amine antiport system is not responsible for the apical uptake of metformin. The present findings may provide new insight into the dose dependency and interindividual variability of the intestinal absorption and bioavailability of metformin.

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