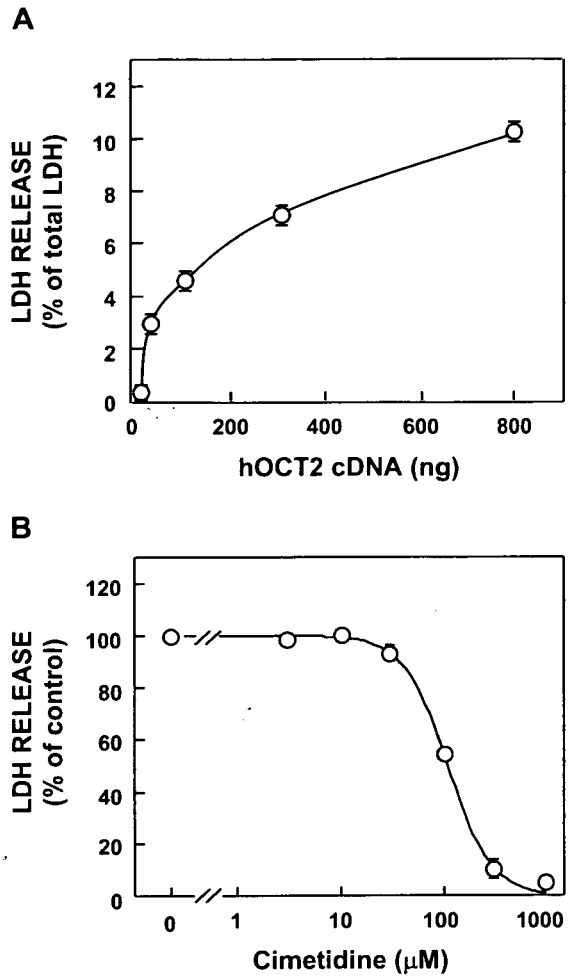

Fig. 2. Role of hOCT2 expression in the cisplatin-induced cytotoxicity. A, HEK293 cells were transfected with each amount of hOCT2 cDNA and vector plasmid added to 800 ng using 2 μl of Lipofectamine 2000. The cells were exposed to 500 μM cisplatin in the medium for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. B, HEK293 cells were transfected with each amount of 100 ng of hOCT2 cDNA and 700 ng of vector plasmid. The cells were incubated in the medium containing 500 μM cisplatin with 0 to 1000 μM cimetidine for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. Each point represents the mean ± S.E.M. of three wells.

Effects of Expression of Basolateral Organic Ion Transporters, hOCT1, hOCT2, hOCT3, hOAT1, and hOAT3, on the Cisplatin-Induced Cytotoxicity in HEK293 Cells. We examined the effect of the basolateral organic ion transporter expression on cisplatin-induced cytotoxicity. When the cells transiently expressing hOCT1, hOCT2, and hOCT3 were treated with 50 to 1000 μM cisplatin for 2 h, the cisplatin-induced cytotoxicity was strongly enhanced by the expression of hOCT2, weakly by the expression of hOCT1, but not by hOCT3 (Fig. 3A). When the cells were transfected with 100 or 800 ng of hOAT1 or hOAT3 cDNA, the cytotoxicity of hOAT-expressing cells was not induced by 500 μM cisplatin (Fig. 3D). In addition, these transporter activities were confirmed by the uptake of their typical substrates, TEA, MPP, PAH, and ES, as shown in Fig. 3, B, C, E, and F.

Effects of hOCT1, hOCT2, and hOCT3 Expression on Platinum Agent-Induced Cytotoxicity in HEK293 Cells. The effects of hOCT1, hOCT2, and hOCT3 expression on the platinum agent-induced cytotoxicity were simultaneously examined using the same passage cells in Fig. 4. When the cells transfected with 100 ng of hOCT1, hOCT2, and hOCT3 were treated with 50 to 1000 μM cisplatin, the highest LDH release in hOCT2-expressing cells among these OCT-expressing cells was observed. Moreover, hOCT1 also enhanced the cisplatin-induced cytotoxicity at higher concentrations, but not hOCT3 (Fig. 4A). The reproducibility (Figs. 3A and 4A) was confirmed in these transfectants, and then we compared the susceptibility of these transfectants to analogs of cisplatin. Oxaliplatin also induced cytotoxicity in HEK293 cells expressing hOCT2 and hOCT3 (Fig. 4C). The treatment with carboplatin and nedaplatin (50–1000 μM) did not affect the LDH release in hOCT1-, hOCT2-, and hOCT3-expressing cells (Fig. 4, B and D).

Transport of Platinum Agents by HEK293 Cells Transiently Expressing Basolateral Organic Cation Transporters, hOCT1, hOCT2, and hOCT3. We compared the accumulations of platinum when HEK293 cells transiently expressing hOCT1, hOCT2, and hOCT3 were treated with the medium containing platinum agents for 1 h. The accumulation of platinum after the treatment with cisplatin was increased in a dose-dependent manner and was much higher in hOCT2-expressing cells and weakly higher in hOCT1-expressing cells (Fig. 5A). When cells were treated with oxaliplatin, it was much higher in hOCT2-expressing cells and slightly in hOCT3-expressing cells (Fig. 5C). The accumulation of carboplatin and nedaplatin was not increased by the expression of hOCT1–3 (Fig. 5, B and D).

Inhibitory Effects of Cisplatin and Oxaliplatin on the [^{14}C]TEA Uptake by HEK293 Cells Expressing Basolateral Organic Cation Transporters, hOCT1, hOCT2, and hOCT3. We compared the affinities of cisplatin and oxaliplatin with hOCT1, hOCT2, and hOCT3 at a concentration range between 50 and 5000 μM because of their solubility. The uptake of [^{14}C]TEA by hOCT1- and hOCT2-expressing cells was partially inhibited by cisplatin but not oxaliplatin at a concentration range between 50 and 5000 μM (Fig. 6, A and B). The inhibitory effect of cisplatin on the uptake of [^{14}C]TEA by hOCT3-expressing cells was stronger than that of oxaliplatin (Fig. 6C).

Effects of the Expression of Apical Organic Cation Transporters, hMATE1, hMATE2-K, hOCTN1, and hOCTN2, on the Cisplatin-Induced Cytotoxicity in HEK293 Cells. When the cells transiently expressing hMATE1, hMATE2-K, hOCTN1, and hOCTN2 were treated with 50 to 1000 μM cisplatin for 2 h, the expression of hMATE1, hMATE2-K, hOCTN1, or hOCTN2 did not affect the cisplatin-induced cytotoxicity (Fig. 7, A and B). In addition, these transporter activities were confirmed by the uptake of [^{14}C]TEA as shown in Fig. 7, C and D.

Transport of Platinum Agents by HEK293 Cells Transiently Expressing Apical Organic Cation Transporters, hMATE1, hMATE2-K, hOCTN1, and hOCTN2. The cellular accumulation of platinum was determined in HEK293 cells expressing hMATE or hOCTN when the cells were treated with 500 μM platinum agents for 1 h. The accumulation of cisplatin was enhanced by hMATE1 more than hMATE2-K. On the other hand, the accumulation of oxaliplatin was enhanced by hMATE2-K more than

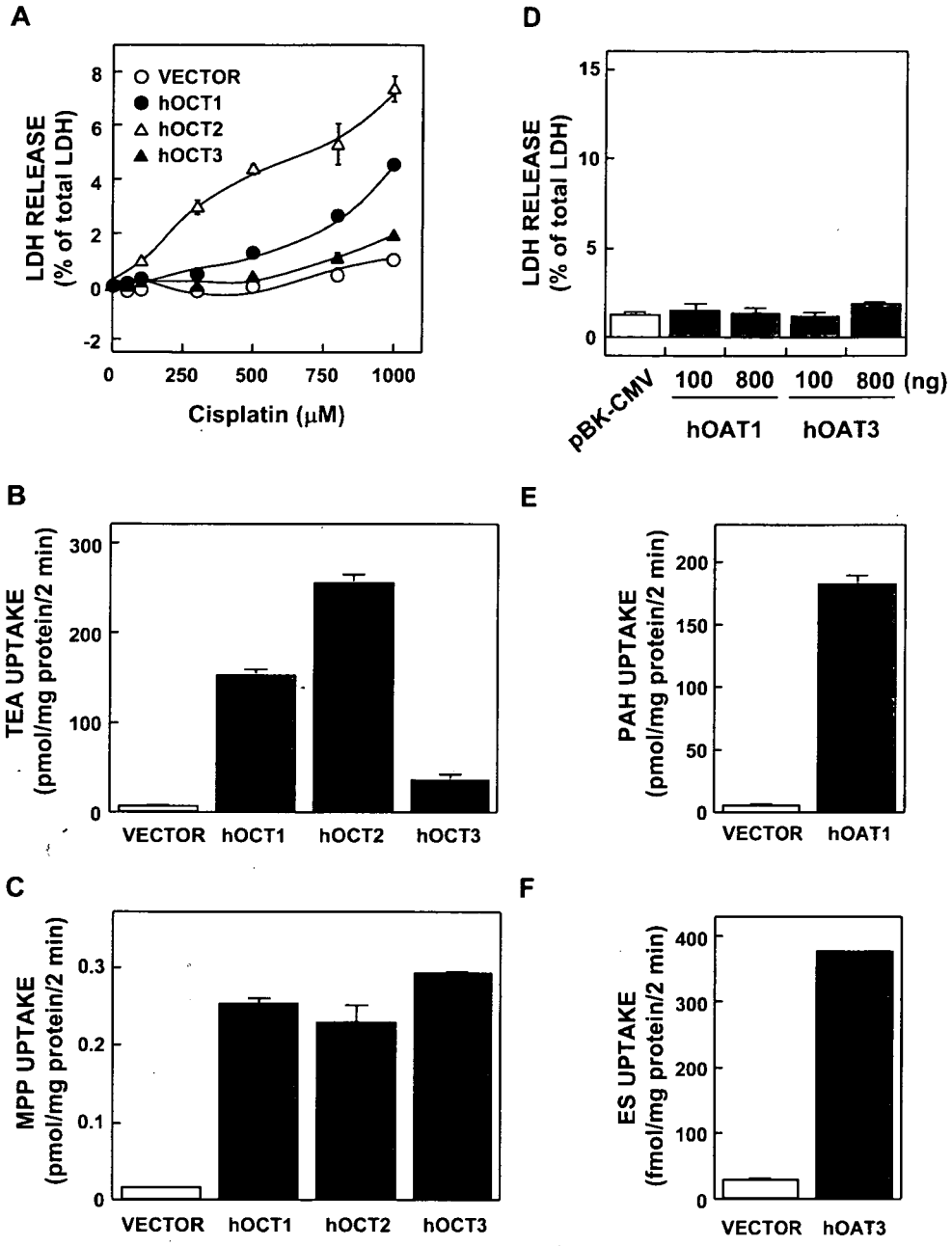

Fig. 3. Effect of basolateral organic ion transporters expression on the cisplatin-induced cytotoxicity. A, HEK293 cells were transfected with empty vector (open circle), hOCT1 (closed circle), hOCT2 (open triangle), or hOCT3 (closed triangle). The cells were treated with the medium containing 50 to 1000 μM cisplatin for 2 h and then incubated in normal medium for 24 h. D, HEK293 cells were transfected with empty vector, hOAT1, and hOAT3 (100 or 800 ng/well). The cells were treated with the medium containing 500 μM cisplatin for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. Uptake of typical substrates, TEA, MPP, PAH, and ES, by HEK293 cells expressing hOCT1, hOCT2, hOCT3, hOAT1, and hOAT3 is shown. HEK293 cells were transfected with empty vector, hOCT1–3 (B and C), hOAT1 (E), or hOAT3 (F). The cells were incubated with the incubation buffer containing 50 μM [^{14}C]TEA (B), 13.7 nM [^{3}H]MPP (C), 5 μM [^{14}C]PAH (E), or 20 nM [^{3}H]ES (F) at pH 7.4 for various periods at 37°C. The amount of substrates in the cells was determined by measuring the radioactivity of solubilized cells. Each point and each bar represent the mean ± S.E.M. of three wells.

hMATE1. These transporters could transport cisplatin and oxaliplatin in culture medium without an artificial pH gradient (Fig. 8A). hOCTN1 or hOCTN2 did not stimulate the transport of platinum agents (Fig. 8B).

Inhibitory Effects of Cisplatin and Oxaliplatin on [^{14}C]TEA Uptake by HEK293 Cells Expressing hMATE1 and hMATE2-K. To examine the uptake of [^{14}C]TEA by hMATE1- and hMATE2-K-expressing cells, the cells were preincubated in the buffer containing ammonium chloride (30 mM) to make an artificial pH-gradient and then were incubated in the buffer, pH 7.4, containing 50 μM [^{14}C]TEA with or without cisplatin or oxaliplatin for 2 min. Cisplatin and oxaliplatin similarly inhibited the uptake of [^{14}C]TEA by hMATE1-expressing cells (Fig. 9A). On the other hand, cisplatin did not inhibit the uptake of [^{14}C]TEA by HEK293 cells expressing hMATE2-K, although oxaliplatin did (Fig. 9B).

Discussion

We previously reported that rOCT2 was responsible for the cisplatin-induced renal tubular toxicity (Yonezawa et al., 2005). In that report, the expression of rOCT2 enhanced the cisplatin-induced cytotoxicity in HEK293 cells, and rOCT2 played a role in the tissue distribution of cisplatin and the cisplatin-induced renal tubular toxicity in the rat. In the present study, we progressively indicated that hOCT2 also abundantly transported oxaliplatin as well as cisplatin, but not carboplatin and nedaplatin (Figs. 4 and 5). In addition, cisplatin and oxaliplatin were transported by hOCT1 and hOCT3, respectively. These results suggested that the basolateral hOCTs are the determinants for the tissue distribution of platinum agents from the circulation. Moreover, apical transporter hMATE1 and hMATE2-K also mediated the transport of cisplatin and oxaliplatin (Fig. 8A). This is the

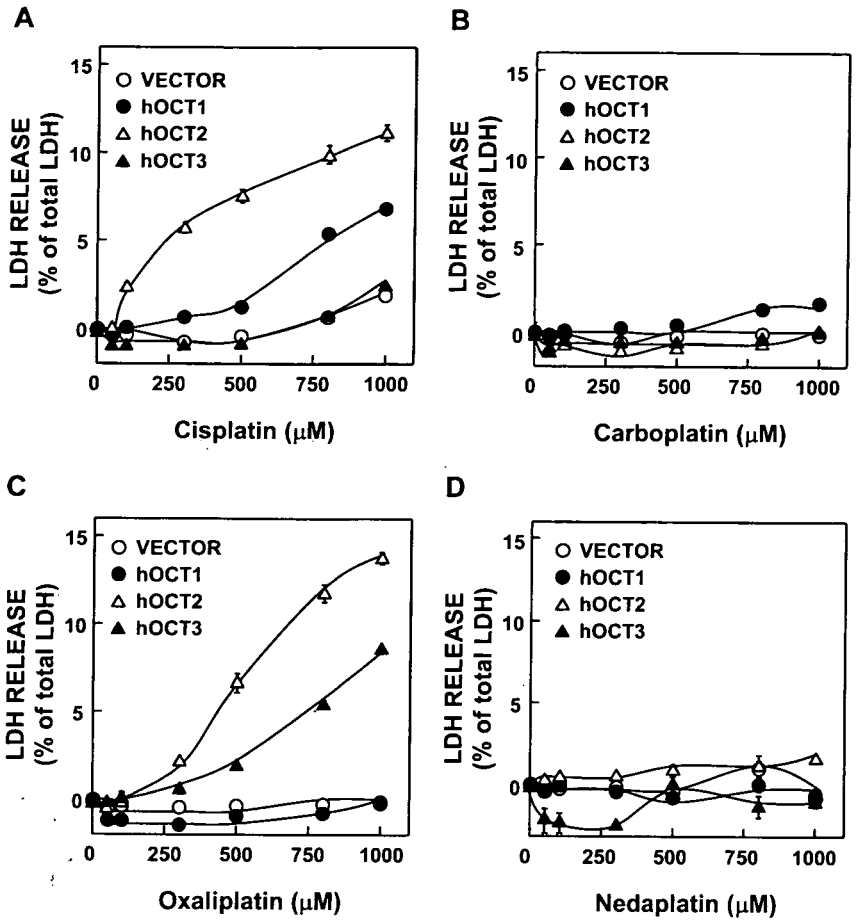

Fig. 4. Effect of hOCT1–3 expression on the platinum agent-induced cytotoxicity. HEK293 cells were transfected with empty vector (open circle), hOCT1 (closed circle), hOCT2 (open triangle), or hOCT3 (closed triangle). The cells were treated with the medium containing 50 to 1000 μM cisplatin (A), carboplatin (B), oxaliplatin (C), and nedaplatin (D) for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. Each point represents the mean ± S.E.M. of three wells.

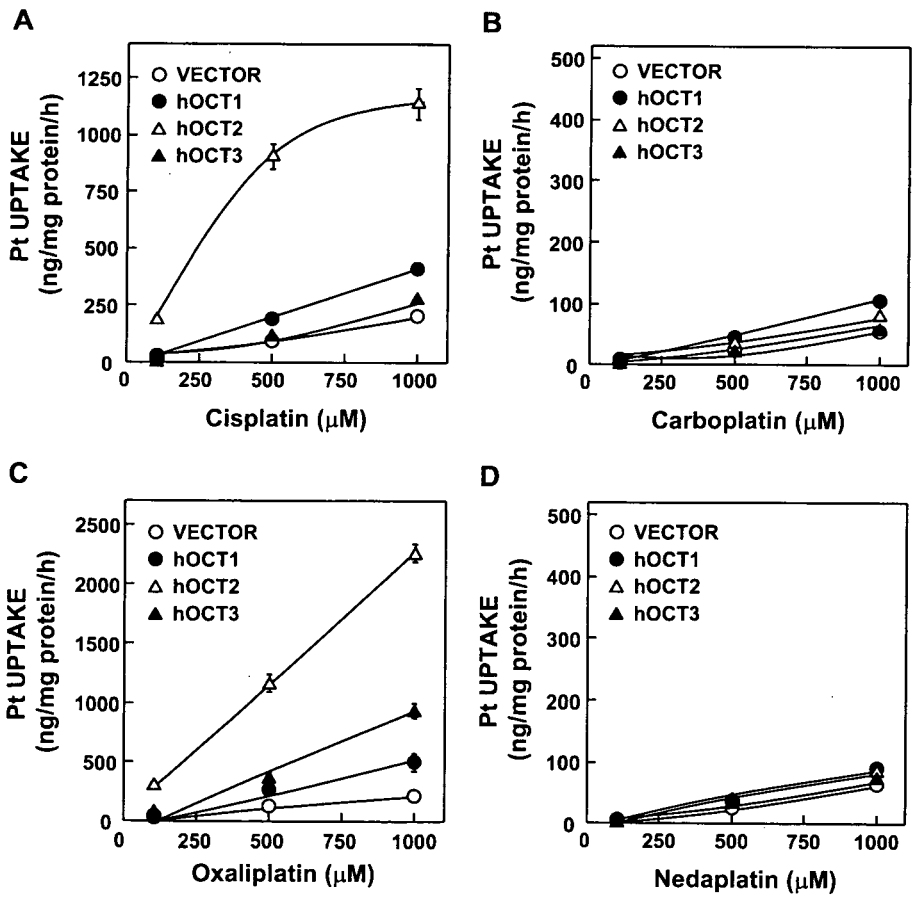

Fig. 5. Uptake of platinum agents by HEK293 cells expressing hOCT1, hOCT2, and hOCT3. HEK293 cells were transfected with empty vector (open circle), hOCT1 (closed circle), hOCT2 (open triangle), or hOCT3 (closed triangle). The cells were treated with the medium containing 100, 500, and 1000 μM cisplatin (A), carboplatin (B), oxaliplatin (C), and nedaplatin (D) for 1 h. After washing, the cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined by ICP-MS. Each point represents the mean ± S.E.M. of four wells.

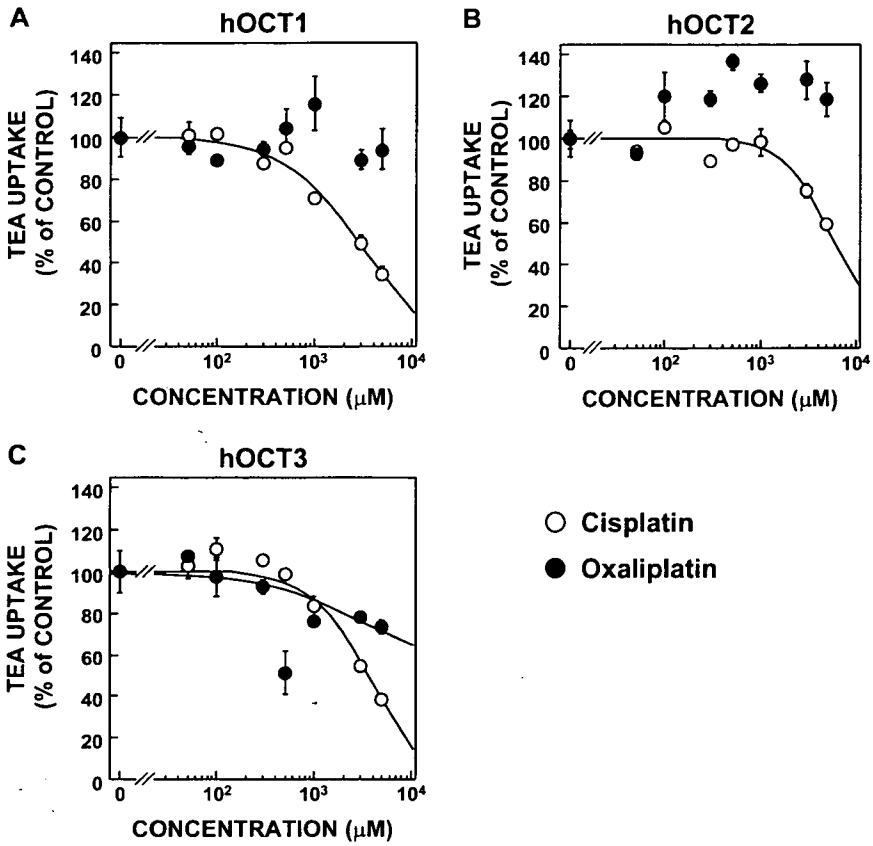

Fig. 6. Inhibitory effects of cisplatin and oxaliplatin on the uptake of [^{14}C]TEA by HEK293 cells expressing hOCT1 (A), hOCT2 (B), and hOCT3 (C). The cells were preincubated in the incubation buffer for 10 min and then were incubated in the buffer containing 50 μM [^{14}C]TEA for 2 min with or without cisplatin and oxaliplatin at various concentrations. The amount of [^{14}C]TEA in the cells was determined by measuring the radioactivity of solubilized cells. Each point represents the mean ± S.E.M. of three wells.

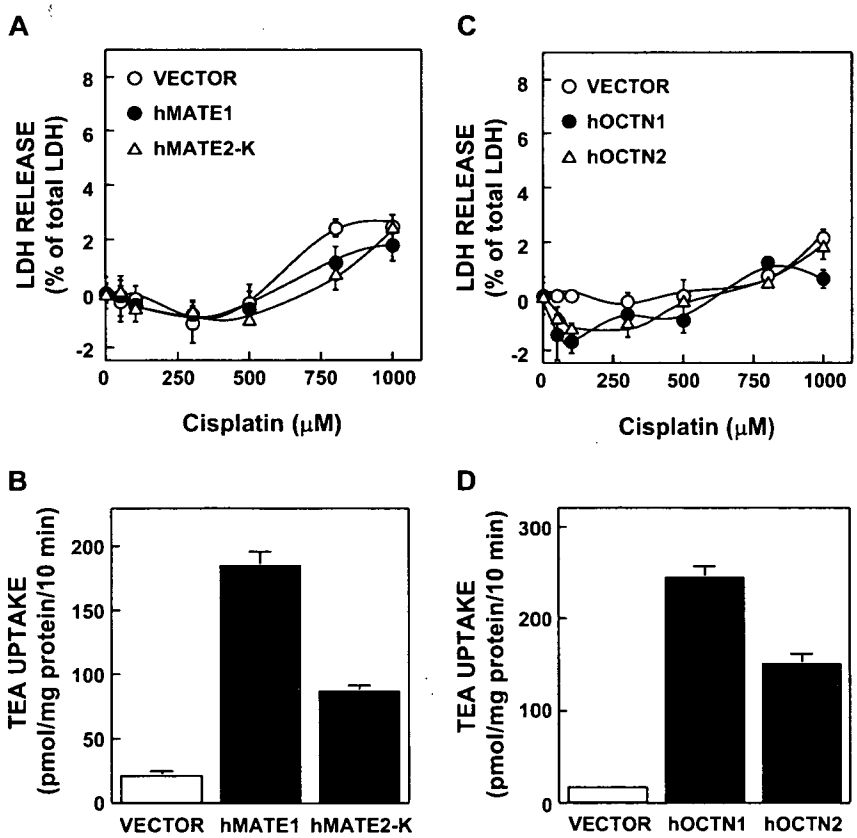

Fig. 7. Effect of apical organic cation transporters expression on cisplatin-induced cytotoxicity. A, HEK293 cells were transfected with empty vector (open circle), hMATE1 (closed circle), or hMATE2-K (open triangle). B, HEK293 cells were transfected with empty vector (open circle), hOCTN1 (closed circle), and hOCTN2 (open triangle). The cells were treated with the medium containing 50 to 1000 μM cisplatin for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. The uptake of typical substrates by HEK293 cells expressing hMATE1, hMATE2-K, hOCTN1, and hOCTN2. HEK293 cells were transfected with empty vector, hMATE1, or hMATE2-K (C) and hOCTN1 or hOCTN2 (D). The cells were incubated with the incubation buffer at pH 8.4 (C) or 7.4 (D) containing 50 μM [^{14}C]TEA for 10 min at 37°C. The amount of substrates in the cells was determined by measuring the radioactivity of solubilized cells. Each point and each bar represent the mean ± S.E.M. of three wells.

first report indicating that cisplatin was transported by hOCT1, hMATE1 and hMATE2-K, as well as hOCT2, and oxaliplatin was transported by hOCT2, hOCT3, hMATE1, and hMATE2-K.

Cisplatin is commonly used against hepatocellular carcinoma, administered from a catheter in the hepatic artery. The systemic blood concentration of cisplatin after its administration from the hepatic artery was not different from that

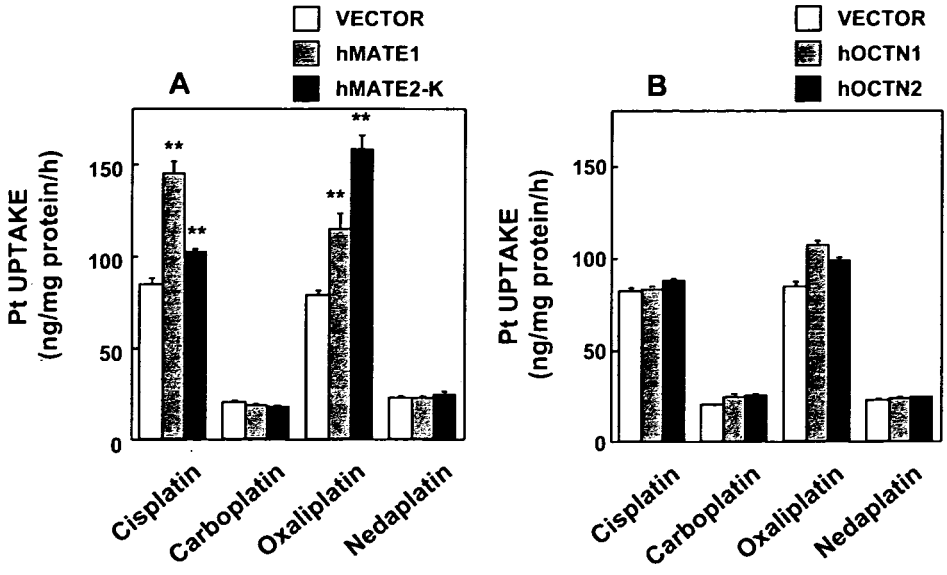

Fig. 8. Uptake of platinum agents by HEK293 cells expressing hMATE1, hMATE2-K, hOCTN1, and hOCTN2. A, HEK293 cells were transfected with empty vector (open bar), hMATE1 (gray bar), and hMATE2-K (black bar). B, HEK293 cells were transfected with empty vector (open bar), hOCTN1 (gray bar), and hOCTN2 (black bar). The cells were treated with the medium containing 500 μM platinum agents for 1 h. After washing, the cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined by ICP-MS. Each bar represents the mean ± S.E.M. of four wells. **, $P < 0.01$, significantly different from vector-transfected cells.

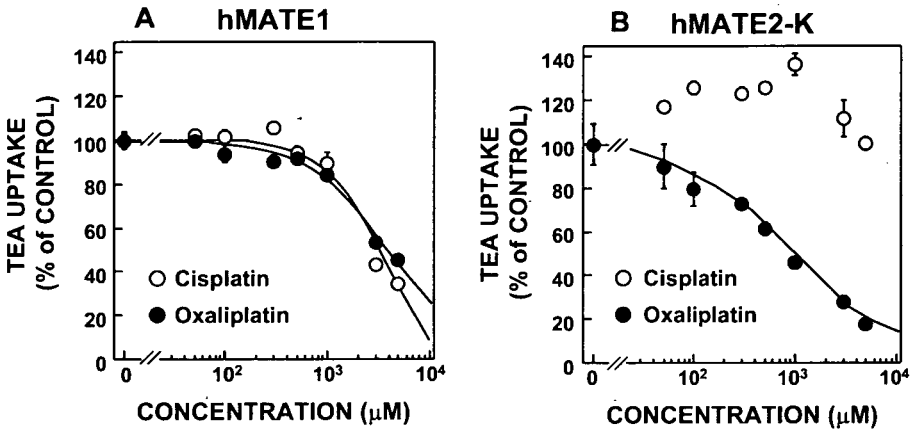

Fig. 9. Inhibitory effects of cisplatin and oxaliplatin on the uptake of [^{14}C]TEA by HEK293 cells expressing hMATE1 (A) and hMATE2-K (B). The cells were preincubated in the incubation buffer containing 30 mM ammonium chloride for 20 min and then were incubated in the buffer containing 50 μM [^{14}C]TEA for 2 min with or without cisplatin and oxaliplatin at various concentrations. The amount of [^{14}C]TEA in the cells was determined by measuring the radioactivity of solubilized cells. Each point represents the mean ± S.E.M. of three wells.

after i.v. administration. Thus, the probability of the adverse effect was not different despite the advantages regarding antitumor efficacy (Lo et al., 2002). It was reported that hepatic OCT1 was expressed in rat diethylnitrosamine-induced hepatocarcinoma cells and differentiated Fao cells, a hepatoma cell line (Lecureur et al., 1998). Interestingly, the cytotoxicity of cisplatin was also enhanced by hOCT1 at a relatively higher concentration (Figs. 3A and 4A). Therefore, hOCT1 was suggested to play an important role in the hepatic uptake of cisplatin.

Oxaliplatin has a different spectrum of activity and the different mechanisms of action and resistance from those of cisplatin and carboplatin (Raymond et al., 2002; Fuertes et al., 2003; Wang and Lippard, 2005). Carboplatin, oxaliplatin, and nedaplatin are low nephrotoxic agents despite their good efficacy. Therefore, it had been assumed that they would not be transported by hOCT2. Expectedly, carboplatin and nedaplatin were not transported by hOCT2 (Figs. 4, B and D, and 5, B and D). Surprisingly, oxaliplatin was transported by hOCT2 (Figs. 4C and 5C), although oxaliplatin did not induce nephrotoxicity (Raymond et al., 2002). In addition, oxaliplatin was transported by hMATE1 and hMATE2-K (Fig. 8A). The nephrotoxic pattern of oxaliplatin observed in the renal slice with collapsed and nonperfused lumens resembled to that of cisplatin (Kanou et al., 2004). It is likely that oxaliplatin would be accumulated in the renal slice without efflux systems, thereby exacerbating its toxicity. Based on these findings and the present results, it is presumed that the basolateral hOCT2 is an influx transporter as the inducer of oxaliplatin-induced toxicity, and the apical hMATE1 and hMATE2-K are efflux transporters as a means to protect cells. Therefore, transcellular transport and cellular toxicity of oxaliplatin should be further examined using polarized double transfectants with both basolateral OCT and apical MATE transporters.

hOCT3 is the organic cation transporter predominantly expressed in the placenta and widely expressed in tissues, including the kidney and intestine (Kekuda et al., 1998). The substrate specificity and pharmacokinetic role of hOCT3 are not fully understood. We identified oxaliplatin as a hOCT3 substrate, but not cisplatin (Fig. 5, A and C). In addition, there is a previous report that oxaliplatin had a more potent cytotoxicity than cisplatin against colon cancer cells, although the molecular mechanism was not clear (Ducreux et al., 1998). Because hOCT3 was reported to be expressed in the intestine (Kekuda et al., 1998), it was supposed that hOCT3 expression would contribute at least in part to the oxaliplatin sensitivity in colon cancer cells.

hMATE1, hMATE2-K, hOCTN1, and hOCTN2 are expressed in the apical membrane of renal proximal tubules. In this study, cisplatin and oxaliplatin were weakly but significantly transported by hMATE1 and hMATE2-K, but not

carboplatin and nedaplatin (Fig. 8A). In contrast, hOCTN1 or hOCTN2 did not transport four platinum agents (Fig. 8B). Because the H^+-gradient stimulated the hMATE1- (Otsuka et al., 2005), hMATE2-K- (Masuda et al., 2006), and rMATE1- (Terada et al., 2006) mediated transport of cationic compounds, the artificial H^+-gradient is necessary as the driving force for the substrate transport. Intracellular acidification by ammonium chloride markedly stimulated the hMATE1-, hMATE2-K-, and rMATE1-mediated uptake of [^{14}C]TEA. As shown in Fig. 8A, the cells were incubated in the culture medium containing platinum agents without an artificial H^+-gradient because of the stability of platinum agents, as described under *Materials and Methods*. It is possible that the transport activities of hMATE1 and hMATE2-K for cisplatin and oxaliplatin are much higher at the renal apical membrane of proximal tubules with an oppositely directed H^+-gradient in the physiological condition.

The clearance of cisplatin was reported to exceed the glomerular filtration rate, suggesting that cisplatin was secreted across the renal tubular cells (Jacobs et al., 1980). In addition, it was reported that cisplatin competitively inhibited the TEA uptake in the basolateral and brush border membrane vesicles (Williams and Hottendorf, 1985), implying that cisplatin was also transported by the organic cation transport system in the basolateral and brush border membrane. In the present study, cisplatin was transported by hOCT2, hMATE1, and hMATE2-K (Figs. 5A and 8A). In addition, the IC_{50} of cisplatin against [^{14}C]TEA uptake by hMATE1-expressing cells was lower than by hMATE2-K-expressing cells (Fig. 9, A and B). It was indicated that cisplatin was a good substrate for hOCT2 and hMATE1 but a low-affinity substrate for hMATE2-K. Therefore, it was suggested that the tubular secretion of cisplatin was explained as follows. Basolateral uptake of cisplatin was mediated by hOCT2 and apical secretion was mediated mainly by hMATE1 and slightly by hMATE2-K. Moreover, carboplatin was reported to not be excreted by the tubular secretion (Sorensen et al., 1992). This report corresponded to our results that carboplatin was not transported by organic cation transporters (Figs. 5B and 8, A and B). Oxaliplatin was transported by hOCT2, hMATE1, and hMATE2-K, but not nedaplatin (Figs. 5C and 8A). The inhibitory effects of oxaliplatin on the hMATE1- and hMATE2-K-mediated transport of [^{14}C]TEA were similar (Fig. 9, A and B). These results suggested that oxaliplatin was secreted across the renal epithelial cells via hOCT2, hMATE1, and hMATE2-K, although nedaplatin was not.

In conclusion, we clearly indicated that cisplatin was transported greatly by hOCT2 and weakly by hOCT1 and that oxaliplatin was transported potently by hOCT2 and weakly by hOCT3. Moreover, cisplatin and oxaliplatin were revealed to be preferentially transported by hMATE1 and hMATE2-K, respectively. These basolateral and apical transporters could play a predominant role in the tissue distribution and tubular secretion of platinum agents, being responsible for anticancer effects and/or adverse effects. Therefore, these transporters may be the molecular targets for the prevention from the resistant and adverse effects in platinum-based chemotherapy.

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Gene expression and regulation of drug transporters in the intestine and kidney☆

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ABSTRACT

Intestinal absorption and renal secretion of ionic drugs are controlled by a number of drug transporters expressed at the brush-border and basolateral membranes of epithelial cells. Over the last several years, considerable progress has been made regarding the molecular identification and functional characterization of drug transporters. Under some physiological and pathophysiological conditions, the expression and transport activity of drug transporters are changed, affecting the pharmacokinetics of substrate drugs. The regulation of transport activity in response to endogenous and exogenous signals can occur at various levels such as transcription, mRNA stability, translation, and posttranslational modification. Transcriptional regulation is of particular interest, because changes in transport activity are dynamically regulated by increases or decreases in levels of mRNA expression. The tissue-specific expression of drug transporters is also under transcriptional control, and recent studies using clinical samples from human tissues have revealed the expression profiles of drug transporters in the human body. The purpose of this research updates is to review the recent progress in the study of the gene expression and regulation of intestinal and renal drug transporters.

1. Introduction

Mucosal surfaces of tissues such as the intestine and kidney are lined by a single layer of epithelial cells. Epithelial cells function as a barrier to select essential (such as nutrients) and waste (such as toxic xenobiotics) compounds, being equipped with uptake and efflux transport systems. During the last decade, many kinds of nutrient and drug transporters in the intestine and kidney have been identified as uptake and efflux transport systems. Currently, various transporters have been classified as ATP-binding cassette (ABC) transporters and solute carriers (SLCs) based on sequence similarity by the Human Gene Nomenclature Committee.

In general, nutrient transporters in the intestine are tightly regulated by nutrient load [1]. Observed patterns of response for essential nutrients and/or nutrients that are toxic in excess, such as zinc and iron, are generally consistent with the maintenance of the body's nutrient status under conditions of

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Abbreviations: ABC, ATP-binding cassette; ATF, activating transcription factor; BCRP, breast cancer resistance protein; Cdx2, caudal-related homeobox protein; CRE, cAMP responsive element; CREB, CRE binding protein; EMSA, electrophoretic mobility shift assay; HNF-4α, hepatocyte nuclear factor-4α; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; OAT, organic anion transporter; OCT, organic cation transporter; OCTN, novel organic cation transporter; PEPT, peptide transporter; Pgp, P-glycoprotein; PKA, protein kinase A; PPARα, peroxisome proliferator-activated receptor α; SLC, solute carrier

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variable intake. For example, expression of the divalent metal ion transporter (DMT1/SLC11A2), involved in iron absorption, is increased in the intestine by an iron-deficient diet [2]. Drug transporters are also regulated by many biochemical signalling pathways, and such regulation may influence the pharmacokinetics of substrate drugs. The regulation of transport activity in response to endogenous and exogenous signals may occur at various levels such as transcription, mRNA stability, translation, and posttranslational modification (Fig. 1). This diversity of regulatory mechanisms may be advantageous to correspond to various biological signals. In general, transcriptional regulation and posttranslational modification are believed to be responsible for long-term and short-term regulation, respectively. We are interested in the transcriptional regulation of drug transporters, because changes in transport activity are dynamically regulated by increases or decreases in levels of mRNA expression. The tissue-specific expression of drug transporters is also under transcriptional control, although there is little information about the mechanisms behind intestinal and renal-specific expression.

This research updates will focus on our current understanding of the expression and gene regulation of drug transporters in the intestine and kidney, concentrating on the control mechanisms governing the expression of each transporter. For SLC drug transporters, H^+/peptide transporters (PEPT) and organic ion transporters (OCT/OCTN/OAT) were selected as representative of transporters expressed in the intestine and kidney. The transporters mainly referred to here are listed in Table 1. On the other hand, only the expression profiles of ABC transporters are covered in this article. We do not refer to the gene regulation of ABC transporters, because several excellent reviews about gene regulation of ABC transporters have been already published [3–6].

2. Function and regulation of drug transporters

2.1. PEPT1 (SLC15A1)

2.1.1. *General function and pharmacokinetic roles*

H^+/peptide cotransporter 1 (PEPT1, SLC15A) is localized at the brush-border membranes of intestinal epithelial cells and plays an important role for protein absorption to mediate the cellular uptake of di- and tripeptides digested from ingested food [7]. Because of its broad substrate specificity, PEPT1 recognizes various peptide-like drugs such as oral β-lactam antibiotics, which are structurally resemble to small peptides [8]. Intestinal PEPT1 can be utilized as a target for improving the intestinal absorption of poorly absorbed drugs such as nucleoside analogues [9,10]. Recently, mathematical models of H^+-coupled substrate transport mediated by PEPT1 were proposed to elucidate the transport characteristics of differently charged substrates [11,12].

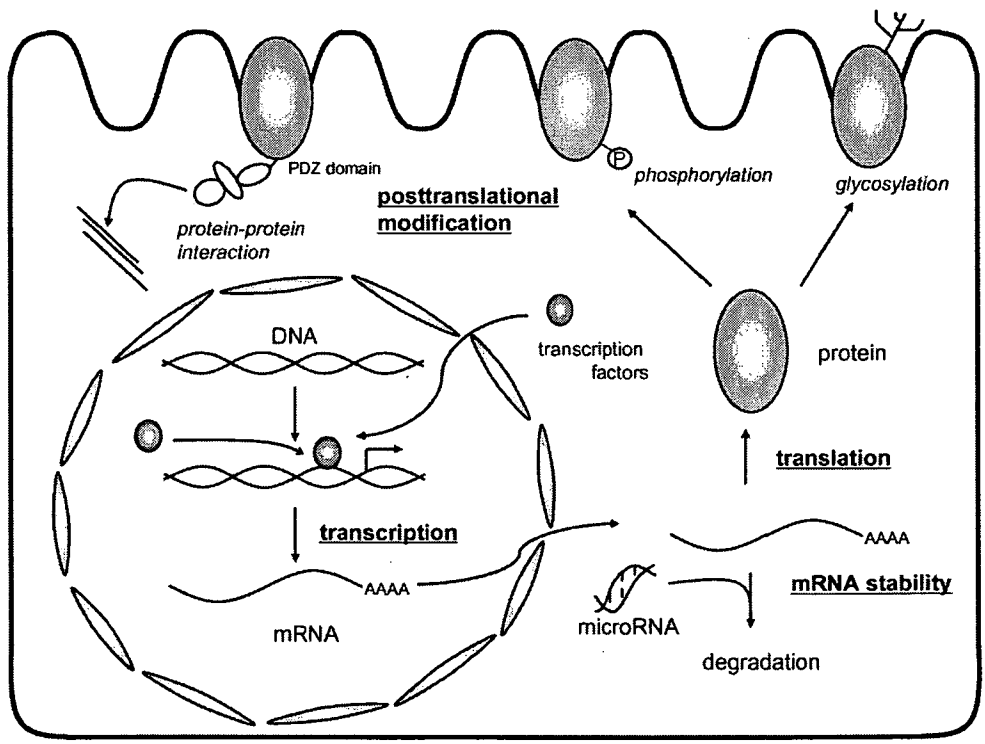

Fig. 1 – Various factors influencing the expression of drug transporters. The activity of drug transporters may be regulated at various levels including transcription, mRNA stability, translation, and posttranslational modification. Posttranslational modification may involve glycosylation, phosphorylation, and protein–protein interaction. Moreover, transcriptional regulation is of particular interest, because many extra- and intracellular signals eventually alter the activity of transcription factors. In addition to the regulation of various signals, the tissue-specific expression of drug transporters is also under transcriptional control.

Table 1 – Major drug transporters expressed in the intestine and kidney

Transporter	Major pharmacokinetic/physiological roles	Tissue distribution	Regulation/diseases	Transcription factors	Reference
PEPT1	Intestinal absorption, renal reabsorption	Small intestine, kidney	Fasting, diurnal rhythm	Sp1	[35]
				Cdx2	[36]
				PPARα	[37]
OCT1	Hepatic uptake	Liver	Intrahepatic cholestasis	HNF-4α	[81]
OCT2	Renal excretion	Kidney	Gender	Androgen receptor (Rat)	[75]
OCTN2	Intestinal absorption, renal reabsorption	Intestine, kidney	Intestinal bowel diseases	Heat-shock transcription factor	[56]
OAT1	Renal excretion	Kidney	Renal failure	Not identified	
OAT2	Hepatic uptake	Liver	Gender	HNF-4α	[82]
OAT3	Renal excretion	Kidney	Renal failure	HNF-1α/β	[79]
				CREB-1	[80]
				ATF-1	[80]

2.1.2. *Regulation of PEPT1*

Adibi [13] summarized the recent progress in the study of the regulation of intestinal PEPT1. He classified the regulation of intestinal PEPT1 as physiological, pathological, or pharmacological regulation. As physiological factors, oligopeptides [14], various hormones (thyroid hormone, insulin, leptin, etc.) [15–17], cytokines (interferon-γ and tumor necrosis factor-α) [18,19], development [20,21], and diurnal rhythm [22–24] regulated the expression of intestinal PEPT1. These regulatory mechanisms vary as follows. In the case of oligopeptides [14], thyroid hormone [17], diurnal rhythm [22–24], and development [20,21], parallel changes in gene expression are brought about by alterations of the transcription and/or stability of PEPT1 mRNA. In contrast, treatment with insulin [15] and leptin [16] did not induce any change in *PEPT1* gene expression, and the mechanism of increased protein expression appears to be increased trafficking from a preformed cytoplasmic pool to the apical membranes. Interferon-γ does not affect the expression of PEPT1 at the mRNA or protein level, but enhances the H^+-electrochemical gradient across the apical plasma membrane in model intestinal epithelial cells [18].

In rats under various nutritional and metabolic conditions (high-protein diet [25,26], fasting [23,25], diabetes [27]), the expression of Pept1 in the intestine was mainly regulated at the transcriptional level. Under the chronic renal failure, intestinal Pept1 expression was regulated at protein level [28]. In patients with intestinal diseases including ulcerative colitis [29], Crohn's disease [29], and short-bowel syndrome [30], PEPT1 expression is induced in the colon. Pharmacological studies have shown that the mRNA expression of PEPT1 can be up-regulated by agents such as pentazocine [31] and 5-fluorouracil [32,33].

2.1.3. *Transcriptional regulatory mechanisms of PEPT1*

Shiraga et al. [26] have revealed that the rat Pept1 promoter was transcriptionally regulated by certain amino acids *via* an amino acid-responsive element. In the mouse Pept1 promoter, essential promoter/enhancer sites were shown to be present within 1140 bp upstream of the transcription start site [34]. Nevertheless, the *cis*-elements and/or transcription factors critical for basal transcriptional regulation have not been identified. To address these issues, we have recently cloned the human PEPT1 promoter region and examined its promoter activity using a human intestinal cell line, Caco-2 [35]. Deletion analysis of the human PEPT1 promoter suggested that the region spanning −172 to −35 bp was essential for basal transcriptional activity. This region lacked a TATA-box but contained some GC-rich sites which supposedly bind with the transcription factor Sp1. Electrophoretic mobility shift assay (EMSA), mutational analysis, inhibition analysis, and overexpression analysis have demonstrated a significant role for Sp1 in the basal transcriptional regulation of PEPT1 [35].

Because Sp1 is ubiquitously expressed, it cannot be the factor responsible for the intestine-specific expression of PEPT1. We then clarified the mechanisms behind the intestinal expression of the *PEPT1* gene [36]. Among the transcription factors investigated, only caudal-related homeobox protein 2 (Cdx2) markedly trans-activated the PEPT1 promoter, although the promoter region responsible for this effect lacked a typical Cdx2-binding sequence, but possessed some Sp1-binding sites. Cdx2 was suggested to have a novel mode of action, namely binding with Sp1. This hypothesis was confirmed by the results of a reporter assay, an immunoprecipitation assay, and a chromatin immunoprecipitation assay. The significance of CDX2 *in vivo* for *PEPT1* regulation was shown by the determination of mRNA levels of CDX2 and PEPT1 in human tissue. In gastric samples, some with intestinal metaplasia, the levels of PEPT1 and CDX2 mRNA were highly correlated (Fig. 2A). These findings collectively suggest that Cdx2 plays a key role in the transcriptional regulation of the intestine-specific expression of PEPT1 through interaction with Sp1 [36] (Fig. 2B).

Starvation markedly increased the amount of mRNA and protein of PEPT1 in rats, leading to altered pharmacokinetics of the PEPT1 substrates [23,25], but the mechanisms underlying this augmentation have not been clarified. We recently examined the role of peroxisome proliferator-activated receptor α (PPARα) in the augmentation of PEPT1 expression by fasting [37], because PPARα plays a pivotal role in the adaptive response to fasting in the liver and other tissues. In 48-h fasted rats, the expression level of PPARα mRNA in the small intestine markedly increased, accompanied by an elevation in serum level of free fatty acids, which are endogenous PPARα ligands. Oral administration of a synthetic

PPARα ligand WY-14643 to fed rats increased the mRNA level of intestinal PEPT1. In the small intestine of PPARα null mice, augmentation of PEPT1 mRNA expression during fasting was completely abolished. To test the possibility that PPARα directly regulates the human PEPT1 promoter, we searched for the potential PPAR responsive element (PPRE) in the promoter region up to 10 kb upstream of the transcription start site and found several candidates. However, none of these sites enhanced basal promoter activity in response to WY-14643 treatment in Caco-2 cells. The functional PPRE and/or some other regulatory region related to PPARα may be located in more distal regions or intronic regions. Taken together, these results indicate that PPARα plays critical roles in fasting-induced intestinal PEPT1 expression [37].

2.2. PEPT2 (SLC15A2)

2.2.1. General function and pharmacokinetic roles

PEPT2 (SLC15A2), mainly expressed in the kidney, shows about 50% amino acid identity with PEPT1, and has higher affinity for substrates than PEPT1 [8]: PEPT2 is also expressed in a variety of tissues such as lung, mammary gland and choroids plexus [8], and recent study revealed that PEPT2 function as an uptake system for peptides fragments from neuropeptide metabolism in the enteric nervous system [38]. This transporter mediates the renal reabsorption of di- and tripeptides and peptide-like drugs through glomerular filtration. Pept2$^{-/-}$ mice were viable and without obvious abnormalities of the kidney [39,40], and detailed *in vivo* analyses using these mice demonstrated that Pept2 is the predominant oligopeptide transporter in the kidney [41]. When Pept2$^{-/-}$ mice were fed diets of different protein contents, Pept2$^{-/-}$ mice adapted food intake to dietary protein content with higher consumption rates on low protein and reduced food intake rates on the high-protein diet [42].

2.2.2. Regulation of PEPT2

Little data is available on the regulation of PEPT2 compared with PEPT1. Using a cell culture model, it was found that Pept2 was regulated by intracellular Ca [43] and epidermal growth factor [44]. Hypothyroidism [45] and thyroidectomy [46] of rats resulted in an increased level of renal PEPT2 expression, suggesting that amino acid homeostasis and drug pharmacokinetics are regulated in states of altered thyroid function. Using 5/6 nephrectomized rats, we found that the renal expression of Pept2, not Pept1, was selectively up-regulated 2 weeks after surgery [47], but levels of both Pept1 and Pept2 were markedly decreased 16 weeks post-surgery [48]. As described above, rat intestinal Pept1 expression showed a

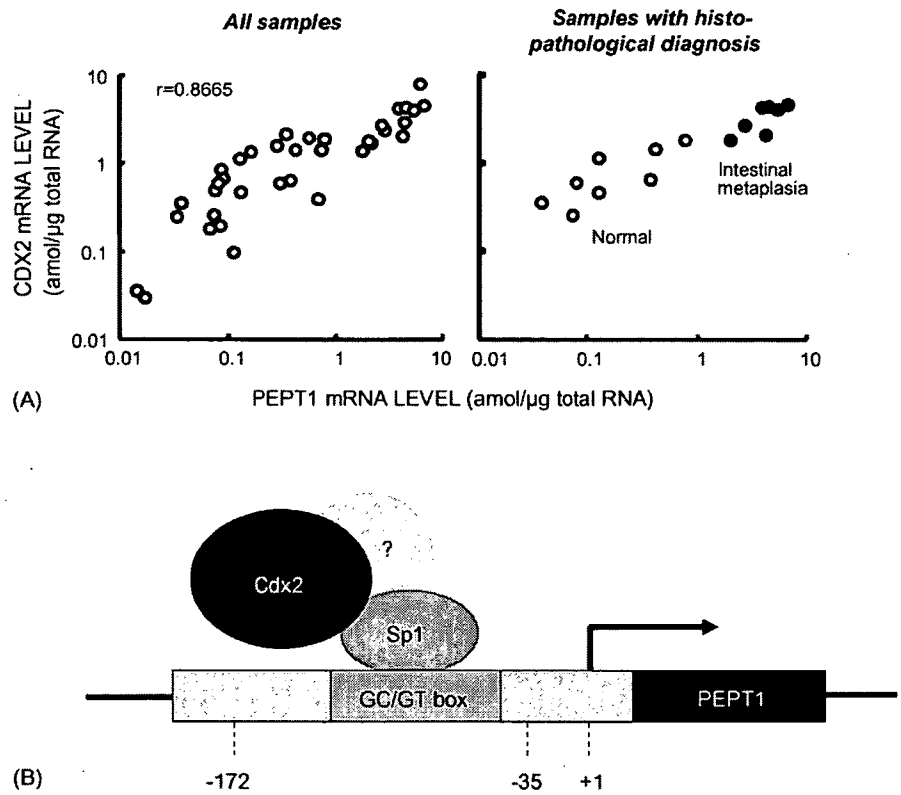

Fig. 2 – Transcriptional regulation of intestinal PEPT1. (A) Correlation between PEPT1 and CDX2 mRNA levels in human gastric tissue samples. The mRNA levels of PEPT1 and CDX2 were quantified with the real-time PCR method in the gastric mucosal samples. Some of these tissue samples were examined by a pathologist who made a diagnosis of intestinal metaplasia. All samples were plotted (left panel). The samples examined by the pathologist were plotted (right panel). Open and closed symbols indicate the normal samples and the samples proved to be case of intestinal metaplasia, respectively [36]. (B) PEPT1 promoter activity is regulated by Cdx2 and Sp1. A reporter assay, immunoprecipitation assay, and chromatin immunoprecipitation assay demonstrated that Cdx2 plays a key role in the transcriptional regulation of the intestine-specific expression of PEPT1 through interaction with Sp1 [35,36].

diurnal rhythm, but renal Pept2 and renal Pept1 expression did not [22]. These findings suggest that the regulatory mechanisms for peptide transporters differ between isoforms and tissues (intestine and kidney).

2.2.3. *Transcriptional regulatory mechanisms of PEPT2*

There is little information available about the transcriptional regulatory mechanisms of PEPT2 except for one report [49]. The mouse *Pept2* gene possesses a TATA-less promoter, and its core promoter was located between 432 and 286 bp upstream from the translation start site.

2.3. *Organic ion transporters (OCT/OCTN/OAT)*

2.3.1. *General function and pharmacokinetic roles*

The organic ion transporter family (SLC22A) consists of organic cation transporters (OCTs), zwitterion/cation transporters (OCTNs) and organic anion transporters (OATs) [50]. This family plays important roles in the renal secretion and hepatic uptake of various compounds including drugs, toxins and endogenous metabolites (OCTs and OATs) [51,52], and in the intestinal and renal absorption of carnitine (OCTN2) [53]. Mutations of transporters for the SLC22 family are responsible for specific diseases such as "primary systemic carnitine deficiency" (OCTN2) [54], and are linked with rheumatoid arthritis (OCTN1) [55] and Crohn's disease (OCTN2) [56].

2.3.2. *Regulation of organic ion transporters*

Not only the functional characteristics but also the regulational aspects of OCTs have been investigated. Ciarimboli and Schlatter [57] recently summarized how the transport of organic cations is regulated in the short and long term. The short-term regulation is mainly caused by the phosphorylation/dephosphorylation of OCT proteins, which induced a change in substrate affinity or membrane trafficking [57]. In the long-term, the regulation of development [58], hormones [59], chronic renal failure [60], and diabetes [61] were demonstrated to affect the expression of Octs in the kidney. The sex hormonal regulation of rat Oct2 in the kidney has been extensively investigated. Namely, the expression level of rat Oct2, but not Oct1 or Oct3, in the kidney was much higher in males than females [62]. The treatment of male and female rats with testosterone significantly increased rat Oct2 expression in the kidney [59,60], suggesting that testosterone plays a pivotal role in the transcriptional regulation of the rat *Oct2* gene. This gender difference in renal Oct2 expression was also observed in rabbits [63] and mice [64], but not clear in humans. In the liver, it was reported that rat hepatic Oct1, not renal Oct1, is down-regulated by obstructive cholestasis [65]. Mouse hepatic Oct1 is demonstrated to be regulated by peroxisome proliferator agonist receptor-α and -γ [66].

The regulation of OATs, in addition to OCTs, has been well studied. Terlouw et al. [67] have summarized the regulation of renal organic anion transporters under various conditions. Using isolated proximal tubules of rabbit kidney and renal cultured cell lines such as OK, the regulation of Oats by various protein kinases and the signal molecules have been investigated, mainly focusing on the short-term regulation [68–70]. It was demonstrated that Oat1 and/or Oat3 in the rat kidney were regulated by development [58], hyperuricemia [71], bilateral ureteral obstruction [72] and acute biliary obstruction [73]. Human OAT1 is also down-regulated by renal failure [74].

2.3.3. *Transcriptional regulatory mechanisms of organic ion transporters*

As described above, there are various reports about the regulation of OCTs and OATs, but most reports do not address the molecular mechanisms behind the alteration of mRNA expression. To understand the role of testosterone in the gender differences in the expression of renal Oct2 in rats, we performed functional reporter analyses of rat *Oct1–3* genes coexpressed with the androgen receptor [75]. It was found that a physiological concentration of testosterone (~10 nM) specifically enhanced transcription of the *Oct2* gene, but not of the *Oct1* or *Oct3* gene, and that androgen response element (ARE)-1 (−2975 to −2960) and ARE-3 (−1340 to −1325) in the rat Oct2 promoter region would play important roles in the enhanced transcription of *Oct2*.

Among the OCT and OAT families, OAT3 showed the most abundant expression in the human kidney [76], and plays important roles in the renal secretion of anionic cephalosporins [74,77,78]. Recently, Kikuchi et al. [79] and we [80] characterized the basal transcriptional activity of the human *OAT3* gene. The minimal promoter region of human *OAT3* was identified to be located approximately 300 bp upstream of the transcription start site, where there are a canonical TATA box (−32 to −27), a hepatocyte nuclear factor-1 (HNF-1)-binding site (−65 to −53), and a cAMP responsive element (CRE) (−87 to −80). Kikuchi et al. [79] demonstrated that HNF-1α/1β were involved in the basal expression of human OAT3 through the interaction of the HNF-1-binding site using intestinal Caco-2 and hepatic HepG2 cells. On the other hand, using the renal proximal tubular cell line OK, we demonstrated that CRE-binding protein (CREB)-1 and activating transcription factor (ATF)-1 were responsible for basal promoter activity by binding to CRE [80]. Furthermore, the activity of the OAT3 promoter was increased through the phosphorylation of CREB-1 and ATF-1 by treatment with 8-bromoadenosine 3′,5′-cyclic monophosphate, a protein kinase A (PKA) activator [80]. These findings indicate that HNF-1α/1β, CREB-1, and ATF-1 function as constitutive regulators of the human OAT3 gene, and that PKA further stimulated OAT3 gene expression by the phosphorylation of CREB-1 and ATF-1 (Fig. 3).

In contrast to OAT3, other SLC22A members such as OCT1 and OAT2 were primarily expressed in the liver. Although the basal transcriptional mechanism of neither transporter has been clarified, liver-specific transcriptional mechanisms were recently demonstrated. Namely, the human *OCT1* [81] and human *OAT2* [82] genes in the liver are activated by a liver-enriched homodimeric nuclear receptor, hepatocyte nuclear factor-4α (HNF-4α) and suppressed by a bile acid-inducible transcriptional repressor, a small heterodimer partner. The hepatic uptake of OCT1 and OAT2 substrates may be decreased in individuals with diseases associated with elevated intracellular levels of bile acids.

Pharmacogenomic analyses have also found the transcription factors involved in the gene expression of OCTN1 and OCTN2. Namely, single nucleotide polymorphisms (SNP)s in the promoter region (regulatory SNPs; rSNPs) of both transporters are suggested to be associated with chronic inflam-

matory diseases, and promoter regions including rSNPs were demonstrated to be functional binding sites of transcription factors, RUNX1 for OCTN1 [55] and heat-shock transcription factor for OCTN2 [56].

3. Expression profile of human intestinal and renal drug transporters

The development of quantitative real-time PCR techniques has meant that expression levels of drug transporters can be quantitatively determined using a very small amount of tissue sample. Recently, based on these techniques, expression profiles of various genes including those for human drug transporters have been determined using surplus tissue specimens collected during surgery or biopsy.

3.1. *Expression profile of intestinal drug transporters*

For SLC drug transporters, the expression profiles of PEPT1 and organic ion transporters along the human digestive tract were determined using normal portions of mucosal samples from cancer patients treated surgically [83]. PEPT1 mRNA was highly expressed in the small intestine (duodenum > jejunum > ileum) compared to other tissues, and some patients showed a significant level of expression in the stomach. The expressional pattern of PEPT1 in the stomach and histological diagnosis indicated that gastric PEPT1 originated from the intestinal metaplasia. This ectopic expression of PEPT1 is induced by the intestine-specific transcription factor CDX2 as described in Section 2.1.3. There was little expression of organic ion transporters except for OCTN2, which showed similar levels to PEPT1 [83]. Using Oct1 knockout mice, it was demonstrated that Oct1 plays important roles in the intestinal excretion of cationic drugs [84]. However, in the human intestine, there is little expression of OCT1.

Taipalensuu et al. [85] determined mRNA levels of 10 ABC drug transporters in the human jejunum, and found that multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) are more extensively expressed than multidrug resistance protein 1 (MDR1). In contrast, Zimmermann et al. [86] demonstrated that MRP3 among MRP1-5 and MDR1 was the most abundantly expressed in the duodenum and all segments of the colon, and that MDR1 showed the highest level of expression in the terminal ileum. The differences between these two reports may be due to the segment of the human intestine sampled. For the distribution of ABC transporters in the intestine, it was reported that MDR1 mRNA [83] and Pgp [87] levels gradually increased from the duodenum to ileum. MRP2 showed significant expression in the small intestine but hardly any expression in colonic segments [86]. BCRP mRNA expression was maximal in the duodenum and decreased continuously down to the rectum [88]. This expression profile contrasts to that of MDR1 mRNA, suggesting that BCRP and MDR1 complement the transport function of each other along the digestive tract as the substrate specificity of the two transporters overlaps.

3.2. *Expression profile of renal drug transporters*

We also reported expression levels of organic ion transporters in normal sections of renal tissue obtained from seven surgically nephrectomized patients with renal cell carcinoma

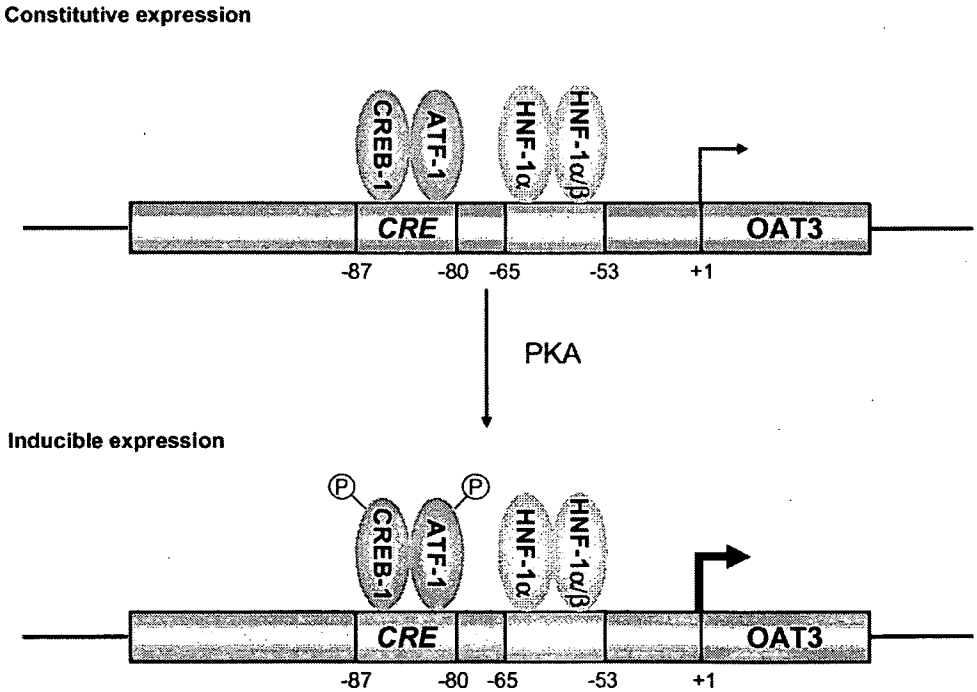

Fig. 3 – Schematic model of transcriptional regulation of the human OAT3 gene. CREB-1 and ATF-1 bind to CRE, and HNF1α/β bind to the HNF1-binding site, and these transcription factors activate the transcription of the OAT3 gene (constitutive expression) [79,80]. PKA further stimulates the transcriptional activity of the OAT3 gene through phosphorylation of both CREB-1 and ATF-1 (inducible expression) [80].

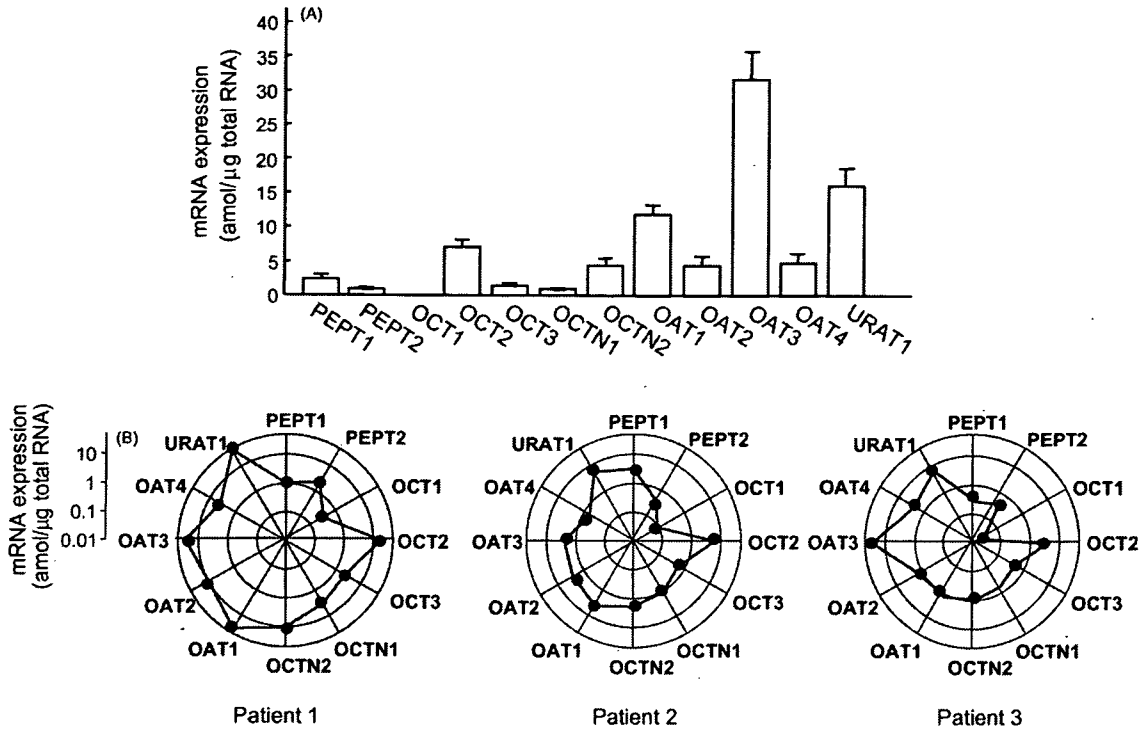

Fig. 4 – Expression profiles of organic ion transporters and peptide transporters in the human kidney cortex. (A) Average expression levels. Each transporter's expression level was determined by the real-time PCR method using a portion of normal human kidney cortex from nephrectomized patients (N = 82). (B) Individual patients' expression profiles (typical patients). Patient 1 shows high levels of OAT1-4 and URAT1, whereas patient 2 exhibits low levels of these transporters, although the expression level of OCT2 was comparable between the two. Patient 3 shows a high level of OAT3, but low levels of other transporters.

in 2002 [76]. Since then, the number of patients has increased to 82, and PEPT1, PEPT2, and an urate transporter (URAT1) have been included in current analyses (Fig. 4A). The mRNA expression level of OAT3 was the highest among the OAT family, followed by that of URAT1, OAT1. The OCT2 mRNA level was the highest in the OCT family, and OCT1 mRNA was rarely expressed. Among the OCTN family, OCTN2 showed the highest expression, but its level was lower than those of OAT1, OAT3, and OCT2. The expression levels of PEPT1 and PEPT2 were not so high as compared to levels of organic ion transporters, and PEPT1 expression was higher than PEPT2 expression. Fig. 4B shows the expression profile of drug transporters for typical individual patients. The expression pattern of drug transporters varied among patients. For example, patient 1 showed high levels of OAT1-4 and URAT1, whereas patient 2 exhibited low levels of these transporters. Regarding the expression of peptide transporters, PEPT1 < PEPT2 for patient 1, PEPT1 > PEPT2 for patient 2, and PEPT1 ≈ PEPT2 for patient 3. Patient 3 showed a high level of OAT3, but low expression levels of other transporters.

4. Conclusions and future perspectives

In these research updates, we addressed recent advances in the study of the gene regulation and expression of drug transporters in the intestine and kidney. Among drug transporters, MDR1 has been well studied in terms of its gene regulation, and many transcription factors for *MDR1* gene have been identified [3,4]. On the other hand, the history of gene regulation for intestinal and renal drug transporters is very short. For example, the transcription factors Sp1, Cdx2 and PPARα were just proven to be responsible for the expression and regulation of intestinal PEPT1, but other transcriptional regulatory mechanisms triggered by various stimuli have not been clarified yet. Information about transcription factors specific to the kidney, especially the proximal tubular cells, is also limited, perhaps due to the absence of an appropriate model of human renal proximal tubular cell lines. Some drug transporters show species differences in renal expression. For example, rat Oct1, but not human OCT1, is expressed in the kidney. The identification of kidney-specific transcription factors should help us to understand not only the molecular mechanisms of such species differences but also the gene regulation of renal drug transporters in various physiological and pathophysiological conditions.

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Oppositely directed H^+ gradient functions as a driving force of rat H^+/organic cation antiporter MATE1

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Tsuda M, Terada T, Asaka J-i, Ueba M, Katsura T, Inui K-i. Oppositely directed H^+ gradient functions as a driving force of rat H^+/organic cation antiporter MATE1. *Am J Physiol Renal Physiol* 292: F593–F598, 2007. First published October 17, 2006; doi:10.1152/ajprenal.00312.2006.—Recently, we have isolated the rat (r) H^+/organic cation antiporter multidrug and toxin extrusion 1 (MATE1) and reported its tissue distribution and transport characteristics. Functional characterization suggested that an oppositely directed H^+ gradient serves as a driving force for the transport of a prototypical organic cation, tetraethylammonium, by MATE1, but there is no direct evidence to prove this. In the present study, therefore, we elucidated the driving force of tetraethylammonium transport via rMATE1 using plasma membrane vesicles isolated from HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells). A 70-kDa rMATE1 protein was confirmed to exist in HEK-rMATE1 cells, and the transport of various organic cations including [^{14}C]tetraethylammonium was stimulated in intracellular acidified HEK-rMATE1 cells but not mock cells. The transport of [^{14}C]tetraethylammonium in membrane vesicles from HEK-rMATE1 cells exhibited the overshoot phenomenon only when there was an outwardly directed H^+ gradient, as observed in rat renal brush-border membrane vesicles. The overshoot phenomenon was not observed in the vesicles from mock cells. The stimulated [^{14}C]tetraethylammonium uptake by an H^+ gradient [intravesicular H^+ concentration ($[H^+]_{in}$) > extravesicular H^+ concentration ($[H^+]_{out}$)] was significantly reduced in the presence of a protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). [^{14}C]tetraethylammonium uptake was not changed in the presence of valinomycin-induced membrane potential. These findings definitively indicate that an oppositely directed H^+ gradient serves as a driving force of tetraethylammonium transport via rMATE1, and this is the first demonstration to identify the driving force of the MATE family. The present experimental strategy is very useful in identifying the driving force of cloned transporters whose driving force has not been evaluated.

multidrug and toxin extrusion 1; transporter; tetraethylammonium; renal secretion; membrane vesicles

THE SECRETION OF DRUGS AND xenobiotics is an important physiological function of the renal proximal tubules. Cationic drugs are secreted from blood to urine by cooperative functions of two distinct classes of organic cation transporters: one driven by the transmembrane potential difference in the basolateral membranes and the other driven by the transmembrane H^+ gradient in the brush-border membranes (7, 16). So far, several membrane potential-dependent organic cation transporters (OCT1–3) have been identified, and their physiological and pharmacokinetic roles have been evaluated (2, 5, 10). However, the molecular nature of H^+/organic cation antiport systems has remained to be elucidated.

Recently, Moriyama and co-workers (3, 15) have identified human (h) and mouse MATE1 and MATE2, which are orthologs of the multidrug and toxin extrusion (MATE) family of bacteria. They demonstrated that MATE1 was predominantly expressed at the luminal membranes of the urinary tubules and bile canaliculi and transported tetraethylammonium, a prototypical organic cation, in a pH-dependent manner (3, 15). We also isolated cDNAs for rat (r) MATE1 (20) and the human kidney-specific isoform MATE2-K (13). rMATE1 was significantly expressed in the kidney and placenta, but not in the liver, and real-time PCR analyses of microdissected nephron segments showed that rMATE1 was expressed in the proximal convoluted and straight tubules (20). On the other hand, hMATE2-K was only expressed in the kidney and was located at the brush-border membranes of renal proximal tubular cells (13). By conducting functional analyses, we showed that rMATE1 and hMATE2-K can transport a wide variety of organic cations including tetraethylammonium, N^1-methylnicotinamide, and metformin (13, 20). These characteristics of MATE1 are similar to those of the H^+/organic cation antiport system revealed by renal brush-border membrane vesicle studies (4, 14, 18, 19, 23).

MATE1 exhibited pH-dependent transport of tetraethylammonium in cellular uptake and efflux studies, and intracellular acidification by NH_4Cl pretreatment stimulated tetraethylammonium transport (3, 13, 15, 20), suggesting that MATE1 utilized an oppositely directed H^+ gradient as a driving force. However, these analyses are not enough to prove the H^+/tetraethylammonium antiport mechanism of MATE1, because it is possible that the pH-dependent transport of tetraethylammonium by MATE1 is regulated not by an H^+ gradient but by pH itself. Accordingly, in addition to the data obtained using the cell culture model, we need more direct evidence that an H^+ gradient is the driving force for MATE1.

In the present study, we developed HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells) and elucidated the driving force of rMATE1 by uptake studies using plasma membrane vesicles from HEK-rMATE1 cells for the first time.

MATERIALS AND METHODS

Materials. [^{14}C]levofloxacin (1.07 GBq/mmol) was kindly provided by Daiichi Pharmaceutical (Tokyo, Japan). [^{14}C]tetraethylammonium bromide (2.035 GBq/mmol), [^{14}C]creatinine (2.035 GBq/mmol), [^{14}C]procainamide (2.035 GBq/mmol), [^{3}H]quinidine (740 GBq/mmol), [^{3}H]quinine (740 GBq/mmol), L-[*N*-methyl-^{3}H]carnitine (3.145 TBq/mmol), and [*N*-methyl-^{14}C]nicotine (2.035 GBq/mmol) were obtained from American Radiolabeled Chemicals (St. Louis,

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MO). [^{14}C]metformin (962 MBq/mmol), [^{14}C]guanidine hydrochloride (1.961 GBq/mmol), [8-^{3}H]acyclovir (110 GBq/mmol), and [8-^{3}H]ganciclovir (370 GBq/mmol) were purchased from Moravec Biochemicals (Brea, CA). [^{3}H]1-methyl-4-phenylpyridinium acetate (2.7 TBq/mmol), [^{3}H]estrone sulfate ammonium salt (2.1 TBq/mmol), and [^{14}C]*p*-aminohippurate (1.9 GBq/mmol) were purchased PerkinElmer Life Analytical Sciences (Boton, MA). [*N*-methyl-^{3}H]cimetidine (451 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). All other chemicals used were of the highest purity available.

Cell culture and transfection. HEK293 cells (American Type Culture Collection CRL-1573) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO_2-95% air at 37°C. pcDNA 3.1 (+) containing cDNA encoding rMATE1 or empty vector was transfected into HEK293 cells using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, the cells were split in complete medium containing G418 (0.5 mg/ml, Nacalai Tesque, Kyoto, Japan) at a dilution of 1:200. Fifteen days after transfection, single colonies were picked out. Cells expressing rMATE1 (HEK-rMATE1 cells) were selected by measuring [^{14}C]tetraethylammonium uptake. Cells transfected with empty vector (HEK-pcDNA cells) were used as controls. These transfectants were maintained in complete medium with G418 (0.5 mg/ml).

Uptake experiments by HEK-rMATE1 cells. The cellular uptake of [^{14}C]tetraethylammonium was measured by using monolayers grown on poly-D-lysine-coated 24-well plates as reported previously with some modifications (13, 20, 22). Briefly, the cells were preincubated with 0.2 ml of incubation medium, pH 7.4 (in mM: 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES) containing 30 mM NH_4Cl for 20 min at 37°C. The medium was then removed, and 0.2 ml of incubation medium (pH 7.4) containing each radiolabeled compound was added. After an appropriate period of incubation, the medium was aspirated, and the monolayers were rapidly washed twice with 1 ml of ice-cold incubation medium (pH 7.4). The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford (1) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine γ-globulin as a standard.

Preparation of membrane vesicles from HEK-rMATE1 cells. Plasma membrane vesicles were prepared according to previous reports (6, 9). HEK-rMATE1 or HEK-pcDNA cells were seeded on 100-mm plastic dishes (4×10^6 cells/dish), and 20 or 40 dishes were used to prepare membrane vesicles in a single preparation. All procedures were performed at 4°C. At the third day after seeding, HEK-rMATE1 or HEK-pcDNA cells were washed with PBS and scraped with a rubber policeman into PBS. The cell suspension was centrifuged at 200 *g* for 10 min, suspended in 20 ml of PBS, and recentrifuged at 200 *g* for 10 min. The packed cell pellet was resuspended in 20 vol of 250 mM mannitol/10 mM HEPES-Tris (pH 7.5)/0.5 mM $MgCl_2$ (*buffer A*), and the cells were gently suspended with five strokes of a loose-fitting Dounce homogenizer. The washed cell suspension was placed in a nitrogen cavitation bomb (Parr Instrument) at 700 lb/in.^{2} for 15 min. After the homogenate was collected, K_2EDTA (pH 7.5) was added to a final concentration of 1 mM. The homogenate was centrifuged at 750 *g* for 15 min, and the supernatant was centrifuged at 20,000 *g* for 15 min. The supernatant was centrifuged at 100,000 *g* for 60 min. The pellet was resuspended in 100 mM mannitol/10 mM MES-KOH (pH 6.0; experimental buffer) or 100 mM mannitol/10 mM HEPES-KOH (pH 7.5; experimental buffer) and centrifuged again at 100,000 *g* for 60 min. The pellet was suspended in the same experimental buffer (pH 6.0 or 7.5) by sucking the suspension 10 times through a fine needle (~4–10 mg protein/ml). KCl (pH 6.0 or 7.5) was added to a final concentration of 100 mM.

Transport experiments by membrane vesicles. The uptake of [^{14}C]tetraethylammonium by membrane vesicles was measured by a rapid filtration technique with a slight modification (8, 19). In the regular assays, the reaction was initiated rapidly by adding 80 μl of buffer, containing 31.25 μM [^{14}C]tetraethylammonium, to 20 μl of membrane vesicle suspension at 25°C. After specified periods, the incubation was terminated by diluting the reaction mixture with 1 ml of ice-cold stop solution containing (in mM) 150 KCl, 20 HEPES-Tris (pH 7.5), 0.1 $HgCl_2$, and 1 tetraethylammonium. The mixture was poured immediately onto Millipore filters (HAWP, 0.45 μm, 2.5 cm in diameter), and the filters were washed with 5 ml of ice-cold stop solution. The radioactivity of [^{14}C]tetraethylammonium trapped in membrane vesicles was determined using an ACS II (Amersham Biosciences) by liquid scintillation counting. The protein content was determined by the method of Bradford (1) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with bovine γ-globulin as a standard.

Western blot analysis. Polyclonal antibody was raised against a synthetic peptide corresponding to the intracellular domain of rMATE1 (CQQAQVHANLKVN, no. 465–477) (13). Brush-border membrane vesicles from rat kidney cortex were prepared as described previously (12). Membrane fractions were separated by SDS-PAGE and analyzed by Western blotting as described previously (17, 21).

Data analysis. Data were analyzed statistically with a one-way analysis of variance followed by Scheffé's test and are expressed as means ± SE.

RESULTS

Generation of HEK-rMATE1 cells. First, we generated and characterized HEK293 cells stably expressing rMATE1. As shown in Fig. 1, an immunoreactive protein with a molecular weight of ~70 kDa was detected in HEK-rMATE1 cells and rat renal brush-border membranes but not in HEK-pcDNA cells. The functional expression of rMATE1 was assessed by measuring the

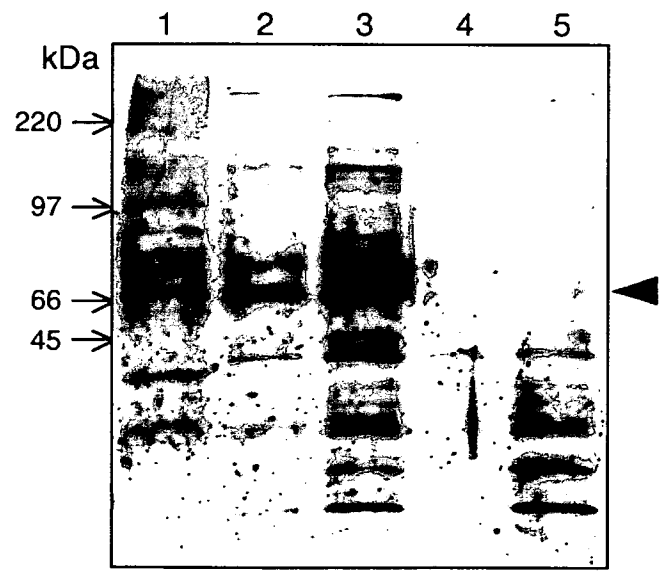

Fig. 1. Western blot analysis of rat renal brush-border membranes and plasma membranes obtained from HEK-rat multidrug and toxin extrusion 1(rMATE1) and HEK-pcDNA cells. Renal brush-border membranes (20 μg) and plasma membranes (5 or 20 μg) obtained from HEK-rMATE1 and HEK-pcDNA cells were separated by SDS-PAGE (10%) and blotted onto polyvinylidene difluoride membranes. The antiserum for rMATE1 (1:1,000) was used as a primary antibody. A horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies, and the strips of blots were visualized by chemiluminescence on X-ray film. The arrowhead indicates the position of rMATE1. Lanes were as follows: *lane 1*, rat renal brush-border membranes; *lane 2*, HEK-rMATE1 (5 μg); *lane 3*, HEK-rMATE1 (20 μg); *lane 4*, HEK-pcDNA (5 μg); and *lane 5*, HEK-pcDNA (20 μg).

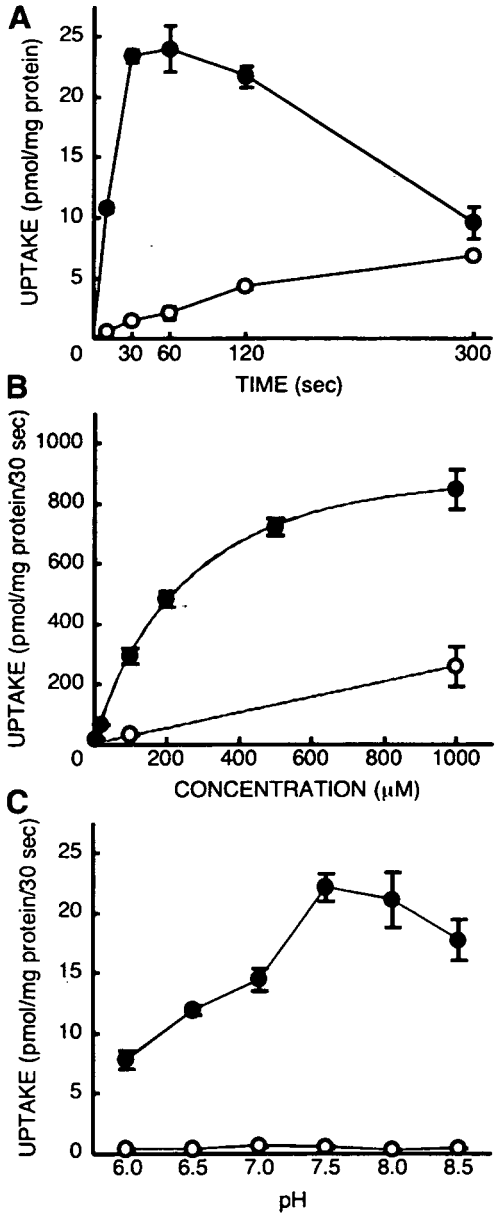

Fig. 2. Transport of [^{14}C]tetraethylammonium (TEA) by HEK-rMATE1 cells. *A*: time course of [^{14}C]TEA uptake by HEK-rMATE1 and HEK-pcDNA cells. HEK-rMATE1 cells (●) and HEK-pcDNA cells (○) were preincubated with 30 mM NH_4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM of [^{14}C]TEA (pH 7.4) for indicated time at 37°C. Each point represents the mean ± SE of 3 monolayers. This figure is representative of 3 separate experiments. *B*: concentration dependence of [^{14}C]TEA uptake by HEK-rMATE1 cells. HEK-rMATE1 cells were preincubated with 30 mM NH_4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with various concentration of [^{14}C]TEA (pH 7.4) in the absence (●) or presence (○) of 5 mM TEA for 30 s at 37°C. Each point represents the mean ± SE of 3 monolayers. *C*: effect of extracellular pH on [^{14}C]TEA uptake by HEK-rMATE1 and HEK-pcDNA cells. HEK-rMATE1 cells (●) and HEK-pcDNA cells (○) were preincubated with 30 mM NH_4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM of [^{14}C]TEA (indicated pH) for 30 s at 37°C. Each point represents the mean ± SE of 3 monolayers. The figure is representative of 2 separate experiments.

uptake of [^{14}C]tetraethylammonium in the HEK-rMATE1 cells under the intracellular acidified conditions caused by NH_4Cl pretreatment. A time- and concentration-dependent uptake of [^{14}C]tetraethylammonium by HEK-rMATE1 cells was observed (Fig. 2, *A* and *B*). [^{14}C]tetraethylammonium uptake by HEK-rMATE1 cells exhibited saturable kinetics, and an apparent K_m value of 304 ± 80 μM was calculated from three separate experiments. When the extracellular pH was changed from 6.0 to 8.5, a bell-shaped pH profile of [^{14}C]tetraethylammonium uptake via rMATE1 was observed, and the uptake was greatest at pH 7.5 and lowest at pH 6.0 (Fig. 2*C*).

Uptake of various compounds by HEK-rMATE1 cells. We then examined the substrate specificity of rMATE1. As shown in Fig. 3, rMATE1 mediated the transport of various organic cations with different chemical structures such as [^{14}C]tetraethylammonium, [^{3}H]1-methyl-4-phenylpyridinium acetate, [^{3}H]cimetidine, and [^{14}C]metformin. The transport of other organic cations such as [^{14}C]procainamide, [^{14}C]creatinine, and [^{14}C]guanidine was greater in HEK-rMATE1 cells than in HEK-pcDNA cells, although the stimulation was not remarkable.

Characteristics of [^{14}C]tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells. Next, we performed transport experiments using plasma membrane vesicles isolated from HEK-rMATE1 cells and HEK-pcDNA cells. In the presence of an H^+ gradient [intravesicular H^+ concentration ($[H^+]_{in}$) > extravesicular H^+ concentration ($[H^+]_{out}$)], a marked

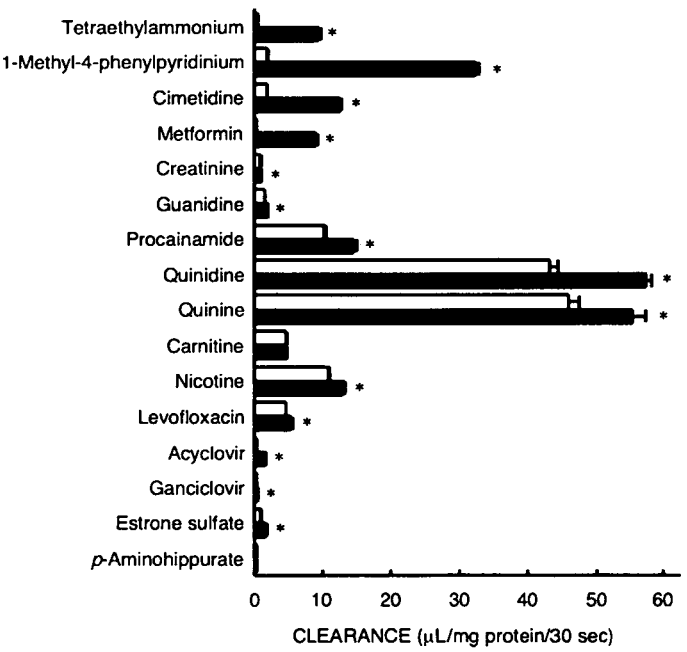

Fig. 3. Uptake of various compounds by HEK-rMATE1 cells. HEK-pcDNA cells (open bars) and HEK-rMATE1 cells (filled bars) were preincubated with 30 mM NH_4Cl (pH 7.4) for 20 min. Then, the preincubation medium was removed, and the cells were incubated with [^{14}C]TEA (5 μM), [^{3}H]1-methyl-4-phenylpyridinium acetate (3.8 nM), [^{3}H]cimetidine (11.1 nM), [^{14}C]metformin (10 μM), [^{14}C]creatinine (5 μM), [^{14}C]guanidine hydrochloride (5 μM), [^{14}C]procainamide (5 μM), [^{3}H]quinidine (13.9 nM), [^{3}H]quinine (13.9 nM), [^{3}H]carnitine (3.3 nM), [^{14}C]nicotine (5 μM), [^{14}C]levofloxacin (14 μM), [^{3}H]acyclovir (92 nM), [^{3}H]ganciclovir (28 nM), [^{3}H]estrone sulfate (4.86 nM), or [^{14}C]*p*-aminohippurate (5 μM) for 30 s at 37°C. Each bar represents the mean ± SE of 3 monolayers. The figure is representative of 2 separate experiments. *$P < 0.05$ significantly different from HEK-pcDNA cells.

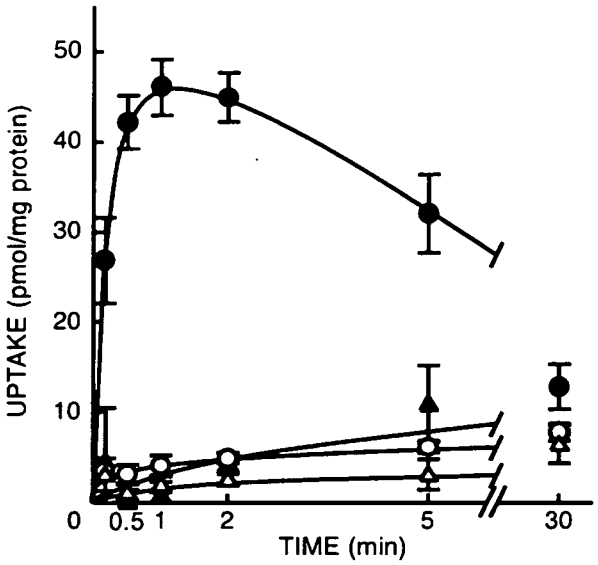

Fig. 4. Time course of [^{14}C]TEA uptake by membrane vesicles from HEK-pcDNA and HEK-rMATE1 cells. The uptake of [^{14}C]TEA by membrane vesicles from HEK-pcDNA cells (○, △) and HEK-rMATE1 cells (●, ▲) was examined in the absence (○, ●) or presence (△, ▲) of 10 mM TEA. Membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [^{14}C]TEA was examined in the experimental buffer containing 31.25 μM [^{14}C]TEA and 100 mM KCl at pH 7.5 in the absence or presence of 10 mM TEA. Each point represents the mean ± SE of 3 determinations.

stimulation of [^{14}C]tetraethylammonium uptake (overshoot phenomenon) was observed in membrane vesicles from HEK-rMATE1 cells, but not in those from HEK-pcDNA cells (Fig. 4). The overshoot phenomenon disappeared in the presence of an excess of cold tetraethylammonium.

Driving force for [^{14}C]tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells. To elucidate the driving force of tetraethylammonium transport by rMATE1, we performed [^{14}C]tetraethylammonium transport experiments using membrane vesicles from HEK-rMATE1 cells. As shown in Fig. 5, the presence of an H^+ gradient ($[H^+]_{in} > [H^+]_{out}$) induced a marked stimulation of [^{14}C]tetraethylammonium uptake against the concentration gradient. On the other hand, no stimulation of [^{14}C]tetraethylammonium uptake was observed in the absence of the gradient or in the presence of the reverse gradient ($[H^+]_{in} < [H^+]_{out}$). The final amount of [^{14}C]tetraethylammonium taken up in the presence of the H^+ gradient ($[H^+]_{in} > [H^+]_{out}$) was not so different from that attained in the absence of the gradient or in the presence of the reverse gradient ($[H^+]_{in} < [H^+]_{out}$).

To further evaluate the effect of an outwardly directed H^+ gradient on [^{14}C]tetraethylammonium uptake, the influence of a protonophore, FCCP, was examined. As shown in Fig. 6*A*, the initial rate of [^{14}C]tetraethylammonium uptake in the presence of an H^+ gradient ($[H^+]_{in} > [H^+]_{out}$) was markedly

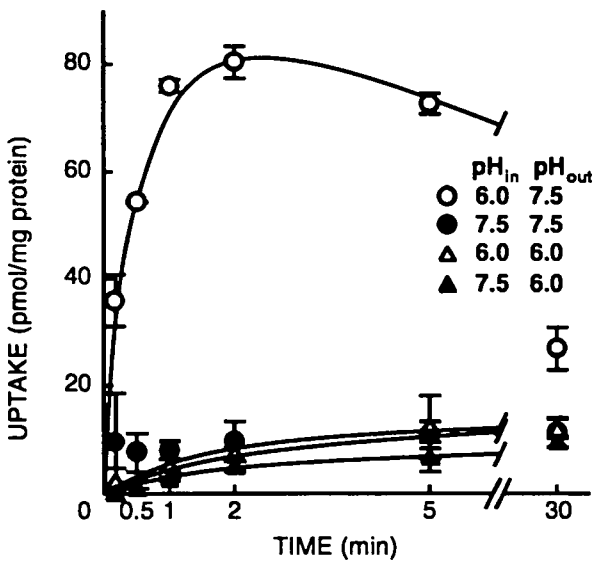

Fig. 5. Effect of H^+ gradient on [^{14}C]TEA uptake by membrane vesicles from HEK-rMATE1 cells. Membrane vesicles were prepared in the experimental buffer at pH 6.0 (○, △) or 7.5 (●, ▲). The uptake of [^{14}C]TEA was examined in the experimental buffer containing 31.25 μM [^{14}C]TEA and 100 mM KCl at pH 6.0 (△, ▲) or 7.5 (○, ●). Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments. pH_{in}, intravesicular pH; pH_{out}, extravesicular pH.

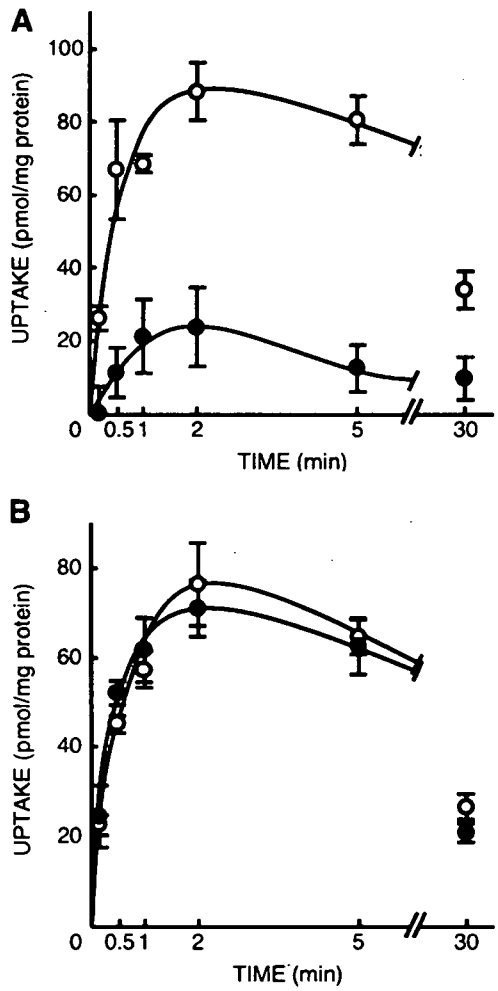

Fig. 6. Effect of FCCP (*A*) and valinomycin (*B*) on [^{14}C]TEA uptake in the presence of an outwardly directed H^+ gradient by membrane vesicles from HEK-rMATE1 cells. *A*: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [^{14}C]TEA was examined in the experimental buffer containing 31.25 μM [^{14}C]TEA and 100 mM KCl at pH 7.5 in the absence (○) or presence (●) of 40 μM FCCP. Each point represents the mean ± SE of 3 determinations. The figure is a representative of 2 separate experiments. *B*: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [^{14}C]TEA was examined in the experimental buffer containing 31.25 μM [^{14}C]TEA and 100 mM CsCl at pH 7.5 in the absence (○) or presence (●) of 8 μM valinomycin. Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments.