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Distinct transport activity of tetraethylammonium from L-carnitine in rat renal brush-border membranes

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Abstract

We investigated the contribution of the Na^+/L-carnitine cotransporter in the transport of tetraethylammonium (TEA) by rat renal brush-border membrane vesicles. The transient uphill transport of L-carnitine was observed in the presence of a Na^+ gradient. The uptake of L-carnitine was of high affinity (K_m = 21 μM) and pH dependent. Various compounds such as TEA, cephaloridine, and *p*-chloromercuribenzene sulfonate (PCMBS) had potent inhibitory effects for L-carnitine uptake. Therefore, we confirmed the Na^+/L-carnitine cotransport activity in rat renal brush-border membranes. Levofloxacin and PCMBS showed different inhibitory effects for TEA and L-carnitine uptake. The presence of an outward H^+ gradient induced a marked stimulation of TEA uptake, whereas it induced no stimulation of L-carnitine uptake. Furthermore, unlabeled TEA preloaded in the vesicles markedly enhanced [^{14}C]TEA uptake, but unlabeled L-carnitine did not stimulate [^{14}C]TEA uptake. These results suggest that transport of TEA across brush-border membranes is independent of the Na^+/L-carnitine cotransport activity, and organic cation secretion across brush-border membranes is predominantly mediated by the H^+/organic cation antiporter.

Keywords: L-Carnitine transporter; Organic cation transporter; Kidney; Brush-border membrane vesicle

1. Introduction

Organic cations are actively secreted by the renal proximal tubules [1]. In general, transport of organic cations in basolateral membranes is facilitated by the transmembrane electrical potential differences, whereas transport of organic cations in the brush-border membrane is mediated by the electroneutral H^+/organic cation antiporter energized by the transmembrane H^+ gradient [2]. The H^+/organic cation antiporter mediates the translocation of various cationic compounds such as tetraethylammonium (TEA) [3,4], cimetidine [5], procainamide [6], neurotoxin 1-methyl-4-phenylpyridinium (MPP) [7] and amino-β-lactam antibiotics [8].

L-Carnitine plays an important role in β-oxidation of long-chain fatty acids [9]. It is usually biosynthesized in some tissues or absorbed from the diet. In the kidney, a high-affinity Na^+/L-carnitine cotransporter expressed in brush-border membranes of proximal tubule cells mediates efficient reabsorption of filtrated L-carnitine (>90%). Thus, the Na^+/L-carnitine cotransporter contributes essentially to maintain the serum level of L-carnitine in circulation [10,11]. Recently, Tamai et al. [12] and Wu et al. [13] identified hOCTN2 as a homolog of hOCTN1 from the human kidney and placenta, respectively. hOCTN2 mediates the high-affinity uptake of L-carnitine in a Na^+-dependent manner [12]. The physiological function of hOCTN2 is suggested to be a high-affinity Na^+/L-carnitine cotransporter in various tissues including the kidney, heart, skeletal muscle, and placenta. Especially in the kidney, hOCTN2 is the key for retaining the serum carnitine concentration. Nezu et al. [14], Tang et al. [15], and Wang et al. [16] reported that the mutations in the hOCTN2 gene cause primary systemic carnitine deficiency, which is characterized by low serum and intracellular concentrations of carnitine. Interestingly, OCTN2 is a Na^+-independent organic cation transporter in addition to being a Na^+-dependent carnitine transporter [17]. Cationic compounds such as TEA, verapamil, and pyrilamine are transported by hOCTN2 in a Na^+-independent manner [18]. Ohashi et al. [19] suggested that OCTN2 might contribute to secretion of the organic cation TEA in the renal apical membrane by comparing the disposition of kidney in wild-type and juvenile visceral steatosis (jvs) mice, which are animal model for systemic carnitine deficiency with mutation of OCTN2. However, the physio-

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logical role of organic cation secretion by OCTN2 in renal brush-border membranes remains to be clarified.

We recently reported that the Na^+/L-carnitine cotransporter is expressed in the apical membranes of LLC-PK_1 cells, the pig kidney epithelial cell line which has been used extensively as a model for the analysis of epithelial functions in renal proximal tubules [20]. The carnitine uptake activity in the apical membrane of LLC-PK_1 cells is functionally similar to OCTN2, a high-affinity Na^+/L-carnitine cotransporter. However, under the conditions in which Na^+/L-carnitine cotransport activity was saturated by adding 100 μM L-carnitine to the apical side medium, the basolateral-to-apical transcellular transport of [^{14}C]TEA was unaffected [20]. These findings suggest that the L-carnitine transport system in the apical membrane of LLC-PK_1 cells is not responsible for the efflux of TEA from the cells. Furthermore, the L-carnitine transport system appeared to be distinct from the H^+/organic cation antiporter, which predominantly mediates the secretion of organic cations [20].

In the present study, to examine whether the H^+/organic cation antiporter or the Na^+/L-carnitine cotransporter mediates predominant secretion of cationic drugs in the renal brush-border membranes, we studied in detail the transport characteristics of L-carnitine and TEA by rat renal brush-border membrane vesicles. The present results suggest that the H^+/organic cation antiporter has distinct characteristics from the Na^+/L-carnitine cotransporter, and that principal secretion of cationic drugs in the renal brush-border membrane is mediated by the H^+/organic cation antiporter.

2. Materials and methods

2.1. Materials

Cephalexin, cephaloridine (Shionogi Co., Osaka, Japan) and levofloxacin (Daiichi Seiyaku Co., Tokyo, Japan) were from the respective suppliers. L-[Methyl-^{3}H]carnitine hydrochloride (3.11 TBq/mmol) was purchased from Amersham International (Buckinghamshire, England). [1-^{14}C]Tetraethylammonium bromide was purchased from Du Pont-New England Nuclear Research Products (Boston, MA). Tetraethylammonium bromide, L-carnitine hydrochloride, cimetidine, and nicotine tartrate dihydrate were purchased from Nacalai Tesque (Kyoto, Japan). 1-Methyl-4-phenylpyridinium iodide was purchased from Research Biochemicals Incorporated (Natick, MA). *p*-Chloromercuribenzene sulfonate (PCMBS) was purchased from Sigma Chemical (St. Louis, MO). Diphenhydramine hydrochloride was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). All other chemicals were of the highest purity available.

2.2. Preparation of brush-border membrane vesicles

The brush-border membrane vesicles were isolated from the renal cortex of male Wistar rats (200–240 g) by the Mg^{2+} precipitation method as described previously [21]. The isolated membrane vesicles were suspended in an experimental buffer to give a final protein concentration of 10 mg/ml. In general, the experimental buffer consisted of either 100 mM mannitol, 100 mM potassium chloride or sodium chloride, and 10 mM HEPES (pH 7.5) or 100 mM mannitol, 100 mM potassium chloride or sodium chloride, and 10 mM MES (pH 6.0), and pH was adjusted with KOH or NaOH.

2.3. Uptake studies

The uptake of L-[^{3}H]carnitine and [^{14}C]TEA by brush-border membrane vesicles was measured by rapid filtration technique. Usually, membrane vesicles suspended in an appropriate buffer were preincubated for 10 min at 25 °C before the initiation of uptake. For the measurement of the trans-stimulation effect, the vesicles were preincubated for 60 min at room temperature to equilibrate the vesicles with a compound to be tested. In general, the uptake was initiated by the addition of 80 μl of a buffer containing 125 nM L-[^{3}H]carnitine (final 100 nM) or 1 μM [^{14}C]TEA (final 0.8 μM) to 20 μl of membrane suspension at 25 °C. At the specified periods, the incubation was terminated by diluting the reaction mixture with 1 ml of ice-cold stop solution containing 150 mM NaCl and 20 mM HEPES/Tris (pH 7.5). The mixture was poured immediately onto Millipore filters (HAWP, 0.45 μm, 2.5 cm diameter), and the filters were washed once with 5 ml of ice-cold stop solution. In separate experiments, the nonspecific association of the ligand with vesicles was estimated by the addition of substrate mixture to 1 ml of ice-cold stop solution containing 20 μl of membrane vesicles. This value was subtracted from the uptake data to evaluate the specific uptake of the ligand. The radioactivity of L-[^{3}H]carnitine and [^{14}C]TEA trapped in membrane vesicles was determined in ACS II (Amersham International, Buckinghamshire, UK) by liquid scintillation counting. The protein content was determined by the method of Bradford [22], using a protein assay kit (Bio-Rad, Richmond, CA) with bovine γ-globulin as the standard.

2.4. Statistical analysis

The statistical significance of differences between mean values was calculated using the non-paired *t* test. Multiple comparisons were performed using Scheffé's test.

3. Results

3.1. Characteristics of L-carnitine uptake by rat renal brush-border membrane vesicles

First, we examined the effect of Na^+ gradient on L-carnitine uptake. The initial rate of L-carnitine uptake was markedly stimulated in the presence of a Na^+ gradient compared with that in the absence of a Na^+ gradient,

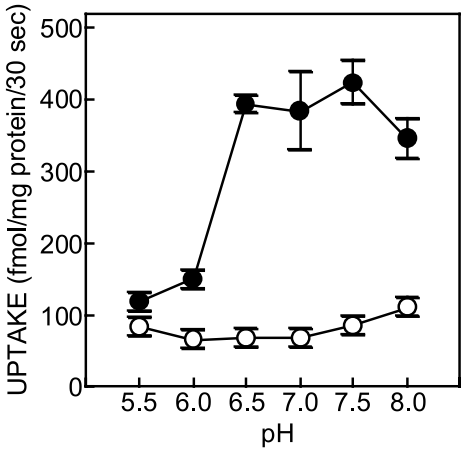

Fig. 1. pH dependence of L-carnitine uptake by rat renal brush-border membrane vesicles. Membrane vesicles (20 μl) suspended in 100 mM mannitol, 100 mM KCl, and 10 mM HEPES (pH 7.5) were incubated at 25 °C for 30 s with the substrate mixture (80 μl) comprised of 100 mM mannitol, 10 mM HEPES (pH 6.5–8.0) or MES (pH 5.5, 6.0), and 100 mM NaCl in the presence (open symbols) or absence (closed symbols) of 1 mM L-carnitine. Each point represents the mean ± S.E. of three determinations.

showing transient uphill transport (overshoot phenomenon).

The concentration dependence of L-carnitine uptake by rat renal brush-border membrane vesicles was also examined. The specific uptake was calculated by subtracting the nonspecific uptake from the total uptake. With the use of nonlinear-least squares regression analysis, kinetic parameters were calculated according to the Michaelis–Menten equation. The apparent Michaelis–Menten constant (K_m) value and maximal uptake rate (V_{max}) value for L-carnitine uptake were 21 μM and 22 pmol/mg protein/15 s, respectively.

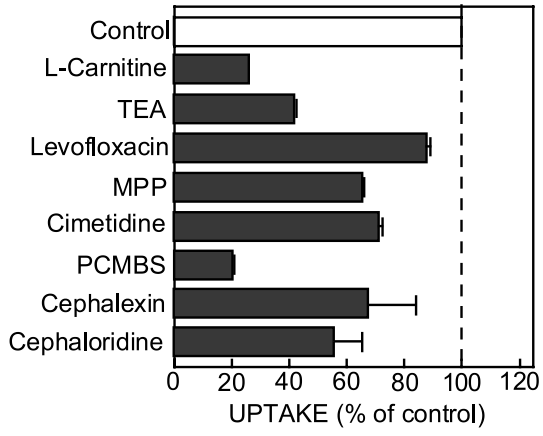

Fig. 2. Effects of various compounds on L-carnitine uptake by rat renal brush-border membrane vesicles. Membrane vesicles (20 μl) suspended in 100 mM mannitol, 100 mM KCl, and 10 mM HEPES (pH 7.5) were incubated at 25 °C for 30 s with the substrate mixture (80 μl) comprised of 100 mM mannitol, 10 mM HEPES (pH 7.5), and 100 mM NaCl in the presence of various compounds (1 mM). Each point represents the mean ± S.E. of three determinations.

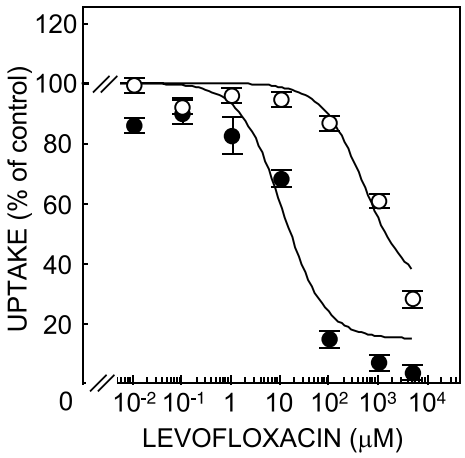

Fig. 3. Effect of levofloxacin on TEA and L-carnitine uptake by rat renal brush-border membrane vesicles. Membrane vesicles (20 μl) suspended in 100 mM mannitol, 100 mM KCl, and 10 mM MES (pH 6.0) or HEPES (pH 7.5) were incubated at 25 °C for 10 or 15 s with the substrate mixture (80 μl) comprised of 100 mM mannitol, 10 mM HEPES (pH 7.5), 100 mM KCl or NaCl, and 1 μM [^{14}C]TEA (closed symbols) or 125 nM L-[^{3}H]carnitine (open symbols) in the presence of various concentrations of levofloxacin. Each point represents the mean ± S.E. of three determinations.

Fig. 1 illustrates the pH dependence of L-carnitine uptake. When the pH in the uptake buffer was acidic, L-carnitine uptake was markedly decreased compared with that at neutral or alkaline pH. However, the uptake at neutral pH was comparable with that at alkaline pH.

3.2. Inhibition of L-carnitine uptake by various compounds

The effects of various compounds on L-carnitine uptake by rat renal brush-border membrane vesicles were exam-

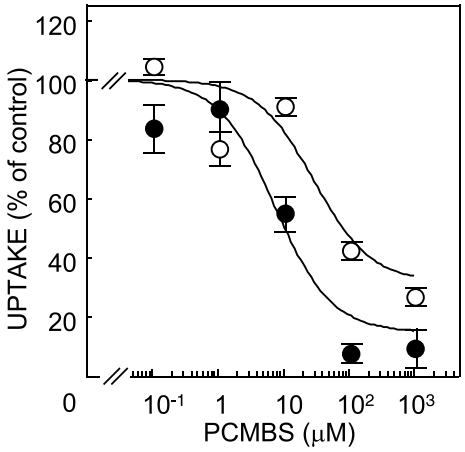

Fig. 4. Effect of PCMBS on TEA and L-carnitine uptake by rat renal brush-border membrane vesicles. Membrane vesicles (20 μl) suspended in 100 mM mannitol, 100 mM KCl, and 10 mM MES (pH 6.0) or HEPES (pH 7.5) were incubated at 25 °C for 10 or 15 s with the substrate mixture (80 μl) comprised of 100 mM mannitol, 10 mM HEPES (pH 7.5), 100 mM KCl or NaCl, and 1 μM [^{14}C]TEA (closed symbols) or 125 nM L-[^{3}H]carnitine (open symbols) in the presence of various concentrations of PCMBS. Each point represents the mean ± S.E. of three determinations.

ined. As shown in Fig. 2, L-carnitine uptake was markedly inhibited by TEA, cephaloridine, and PCMBS, whereas the uptake was slightly inhibited by MPP, cimetidine, and levofloxacin.

3.3. Inhibitions of TEA and L-carnitine uptake by levofloxacin and PCMBS

We compared the effect of levofloxacin on TEA and L-carnitine uptake. As shown in Fig. 3, levofloxacin inhibited TEA uptake more strongly than L-carnitine uptake. The IC_{50} values of levofloxacin for TEA and L-carnitine uptake were 6 and 543 μM, respectively. Furthermore, Fig. 4 shows the effect of PCMBS on TEA and L-carnitine uptake. PCMBS potently inhibited both L-carnitine and TEA uptake. The IC_{50} values of PCMBS for TEA and L-carnitine uptake were 4 and 30 μM, respectively.

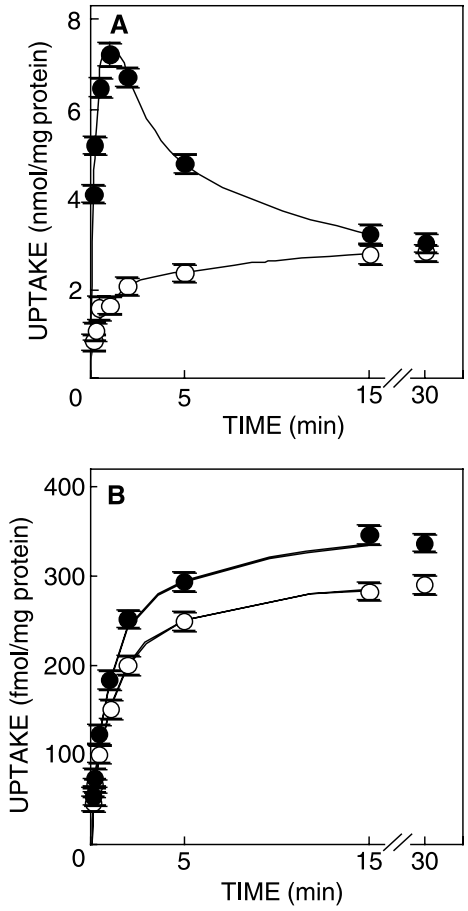

Fig. 5. Effect of H^+ gradient on TEA (A) and L-carnitine (B) uptake by rat renal brush-border membrane vesicles. Membrane vesicles (20 μl) suspended in 100 mM mannitol, 100 mM KCl, and 10 mM MES (pH 6.0, closed symbols) or HEPES (pH 7.5, open symbols) were incubated at 25 °C for 10 s with the substrate mixture (80 μl) comprised of 100 mM mannitol, 10 mM HEPES (pH 7.5), 100 mM KCl, and 1 μM [^{14}C]TEA (A) or 125 nM L-[^{3}H]carnitine (B). Each point represents the mean ± S.E. of three determinations.

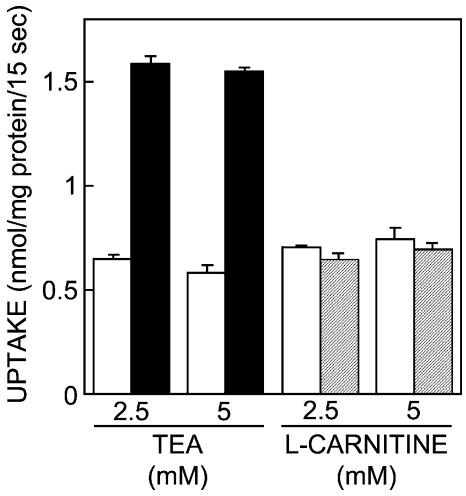

Fig. 6. Trans-stimulation effects of unlabeled TEA and L-carnitine on [^{14}C]TEA uptake by rat renal brush-border membrane vesicles. Membrane vesicles were preincubated at room temperature for 1 h in 100 mM mannitol, 100 mM NaCl, and 10 mM HEPES (pH 7.5) with each concentration of unlabeled TEA (solid columns) or L-carnitine (hatched columns) and then aliquots (20 μl) were incubated at 25 °C for 15 s with the substrate mixture (180 μl) comprised of 100 mM mannitol, 10 mM HEPES (pH 7.5), 100 mM NaCl, and 0.278 μM [^{14}C]TEA. Control (open bar) were shown as [^{14}C]TEA uptake in the presence of extracellular unlabeled TEA and L-carnitine (250 and 500 μM). Each column represents the mean ± S.E. of three determinations.

3.4. Effect of H^+ gradient on TEA and L-carnitine uptake

We examined the effect of an outward H^+ gradient on TEA and L-carnitine uptake. As shown in Fig. 5A and B, TEA

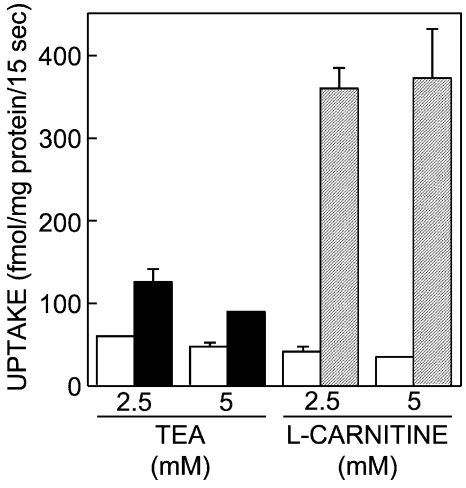

Fig. 7. Trans-stimulation effects of unlabeled TEA and L-carnitine on L-[^{3}H]carnitine uptake by rat renal brush-border membrane vesicles. Membrane vesicles were preincubated at room temperature for 1 h in 100 mM mannitol, 100 mM NaCl, and 10 mM HEPES (pH 7.5) with each concentration of unlabeled TEA (solid columns) or L-carnitine (hatched columns) and then aliquots (20 μl) were incubated at 25 °C for 15 s with the substrate mixture (180 μl) comprised of 100 mM mannitol, 10 mM HEPES (pH 7.5), 100 mM NaCl, and 125 nM L-[^{3}H]carnitine. Control (open bar) were shown as L-[^{3}H]carnitine uptake in the presence of extracellular unlabeled TEA and L-carnitine (250 and 500 μM). Each column represents the mean ± S.E. of three determinations.

uptake was markedly stimulated in the presence of a H^+ gradient, showing transient uphill transport; on the other hand, L-carnitine uptake was not. L-carnitine uptake increased slightly in the presence of an outward H^+ gradient (Fig. 5B).

3.5. Trans-stimulation effects on TEA and L-carnitine uptake

Fig. 6 shows the trans-stimulation effects of unlabeled TEA and L-carnitine on TEA uptake. The initial uptake rate of [^{14}C]TEA was markedly enhanced in the vesicles preloaded with unlabeled TEA, but the uptake was not enhanced by unlabeled L-carnitine. In addition, we examined the trans-stimulation effect of unlabeled TEA and L-carnitine on L-[^{3}H]carnitine uptake. As depicted in Fig. 7, the uptake of L-[^{3}H]carnitine was markedly stimulated in the vesicles preloaded with unlabeled L-carnitine, whereas the uptake was only slightly affected by unlabeled TEA.

4. Discussion

It was previously reported that the transport of organic cations such as TEA and cimetidine in renal brush-border membranes was mediated by the electroneutral H^+/organic cation antiporter energized by a transmembrane H^+ gradient [2]. Recently identified OCTN2 is not only a Na^+-dependent carnitine transporter but also a Na^+-independent organic cation transporter [17,19]. Therefore, to distinguish the H^+/organic cation antiporter from the Na^+/L-carnitine cotransporter on renal secretion of cationic drugs, the present study examined the transport characteristics of L-carnitine and TEA by rat renal brush-border membrane vesicles.

We here demonstrated that the Na^+/L-carnitine cotransport activity, which is similar to that of hOCTN2 in transport characteristics, was expressed on rat renal brush-border membranes. In the presence of a Na^+ gradient, L-carnitine uptake by the brush-border membranes was markedly stimulated, showing a transient uphill transport and our kinetic analysis suggested that L-carnitine uptake was high affinity (K_m = 21 μM) (data not shown). Furthermore, L-carnitine uptake was dependent on the pH of the uptake buffer with lowering uptake at acidic pH (Fig. 1). Tamai et al. [12] demonstrated that hOCTN2 mediated Na^+- and pH-dependent L-carnitine transport with a K_m value of 4.3 μM. In addition, another uptake study using mouse renal brush-border membrane vesicles showed a definite overshoot of Na^+- and concentration-dependent L-carnitine transport with a K_m value of 18.7 μM [23]. The present results appear to be compatible with those, although the precise mechanisms of pH dependence of the Na^+/L-carnitine cotransporter still remain to be clarified. The uptake of L-carnitine was not stimulated in the presence of outward H^+ gradient (Fig. 5B), suggesting that the H^+/L-carnitine exchange mechanism can be most likely ruled out. Therefore, an allosteric regulation of L-carnitine transport by protons might be involved in the decreased uptake [24], because a charge of zwitterionic L-carnitine (pK_a = 3.7) was not changed in the present condition (pH 5.5–8.0).

Ohashi et al. [18] reported that the L-carnitine uptake by hOCTN2 was significantly inhibited by various cationic and zwitterionic compounds. In the present study, the inhibitory effects of various compounds on L-carnitine uptake were comparable with those of Ohashi et al. [18] (Fig. 2). MPP, cimetidine, and the quinolone antibacterial drug levofloxacin moderately inhibited L-carnitine uptake by the brush-border membranes. In contrast, cephalexin showed no inhibitory effect. It was also reported that all of these cationic compounds strongly inhibit the H^+/organic cation antiporter in the renal brush-border membranes [5,8,25,26]. Accordingly, the present results suggest that the substrate specificity of the Na^+/L-carnitine cotransporter in rat renal brush-border membranes is different from that of the H^+/organic cation antiporter.

Okano et al. [27] demonstrated that ofloxacin, an enantiomer of levofloxacin, inhibits about 70% of TEA uptake in rat renal brush-border membrane vesicles. Ohtomo et al. [25] also reported that levofloxacin markedly inhibited the apical H^+/organic cation antiporter in LLC-PK_1 cell monolayers. However, in the present study, 1 mM of levofloxacin could only slightly inhibit the Na^+/L-carnitine cotransporter on rat renal brush-border membranes. Therefore, we compared the affinity of levofloxacin on TEA and L-carnitine uptake by the renal brush-border membrane vesicles. Levofloxacin inhibited TEA uptake more strongly than L-carnitine uptake (Fig. 3), suggesting that levofloxacin interacts preferentially with the H^+/organic cation antiporter rather than the Na^+/L-carnitine cotransporter. Furthermore, we examined the effect of PCMBS on TEA and L-carnitine uptake by the brush-border membranes. We previously reported that PCMBS specifically inhibits the H^+ gradient-dependent transport of TEA in the renal brush-border membranes [28] and LLC-PK_1 cells [29]. In the present study, PCMBS potently inhibited TEA as well as L-carnitine (Fig. 4). Considering the membrane vesicles to be oriented right-side-out, it is suggested that the functional sulfhydryl groups of the H^+/organic cation antiporter and the Na^+/L-carnitine cotransporter could be localized at the luminal surface of the proximal tubule cells.

The uptake of [^{14}C]TEA was stimulated in the presence of an outward H^+ gradient (Fig. 5A), in an agreement with the previously reported transport mechanism of organic cations [2], whereas the uptake of L-[^{3}H]carnitine was not stimulated in the presence of an outward H^+ gradient (Fig. 5B), suggesting that H^+ gradient is not the driving force for L-carnitine uptake by the brush-border membranes. However, it is noteworthy that a slight increase in L-carnitine uptake was observed in the presence of an H^+ gradient (Fig. 5B). This finding suggests that L-carnitine might be partially transported by the H^+/organic cation antiporter. On the other hand, this result could reflect a small inside negative potential generated by an outwardly directed H^+ gradient,

because OCTN2-mediated L-carnitine transport is electrogenic [11,24,30].

Trans-stimulation of [^{14}C]TEA uptake by unlabeled TEA was observed, whereas trans-stimulation by unlabeled L-carnitine was not observed (Fig. 6). This result suggests the predominant transport activity of TEA by the H^+/organic cation antiporter on rat renal brush-border membranes. We also found that the uptake of [^{14}C]TEA was stimulated by unlabeled L-carnitine being preloaded into vesicles in the presence of an outward Na^+ gradient (data not shown). In renal brush-border membranes, the H^+ gradient could be created by a Na^+/H^+ antiporter [31–33] Therefore, the stimulative effect could be due to the two functionally linked antiport system, the Na^+/H^+ antiporter and the H^+/organic cation antiporter. Furthermore, we examined the trans-stimulation effects of L-carnitine uptake. The uptake of L-[^{3}H]carnitine by the renal brush-border membranes was markedly enhanced by unlabeled L-carnitine (Fig. 7). Interestingly, the slight stimulation of L-[^{3}H]carnitine uptake by unlabeled TEA was observed (Fig. 7), suggesting that the Na^+/L-carnitine cotransporter might possibly have a functional property similar to L-carnitine/organic cation exchanger partially in the renal brush-border membranes. Ohashi et al. [19] reported that jvs mice showed decreased renal secretory clearance of TEA, and that mutual trans-effects of OCTN2-mediated TEA and L-carnitine transport were observed in OCTN2-expressing HEK293 cells. Concerning these findings, they proposed that OCTN2 functions as an exchanger of Na^+-dependent L-carnitine reabsorption and Na^+-independent secretion of organic cations in renal epithelial cells [19]. However, they also demonstrated that renal function (glomerular filtration and/or renal blood flow) in jvs mice may be impaired due to sever cardiomyopathy [19]. Taking this finding into consideration, it could be speculated that loss of renal organic cations secretion in jvs mice might reflect not only impairment of OCTN2 as organic cation transporter but also that of other transporters containing H^+/organic cation antiporter. Therefore, the contribution of the Na^+/L-carnitine cotransporter to renal secretion of cationic drugs is not clear. The Na^+/L-carnitine cotransporter is expressed in ubiquitous tissues including the kidney, heart, skeletal muscle, and placenta. It should be further investigated whether the Na^+/L-carnitine cotransporter contributes to the disposition of pharmacologically active cationic drugs in these tissues.

In conclusion, the present results suggest that principal organic cation transport activity is independent of the Na^+/L-carnitine cotransporter in rat renal brush-border membranes, and that the H^+/organic cation antiporter plays a predominant physiological role in organic cation secretion in the proximal tubule cells.

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