

## Na<sup>+</sup>-coupled transport of L-carnitine via high-affinity carnitine transporter OCTN2 and its subcellular localization in kidney

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### Abstract

The mechanism of Na<sup>+</sup>-dependent transport of L-carnitine via the carnitine/organic cation transporter OCTN2 and the subcellular localization of OCTN2 in kidney were studied. Using plasma membrane vesicles prepared from HEK293 cells that were stably transfected with human OCTN2, transport of L-carnitine via human OCTN2 was characterized. Uptake of L-[<sup>3</sup>H]carnitine by the OCTN2-expressing membrane vesicles was significantly increased in the presence of an inwardly directed Na<sup>+</sup> gradient, with an overshoot, while such transient uphill transport was not observed in membrane vesicles from cells that were mock transfected with expression vector pcDNA3 alone. The uptake of L-[<sup>3</sup>H]carnitine was specifically dependent on Na<sup>+</sup> and the osmolarity effect showed that Na<sup>+</sup> significantly influenced the transport rather than the binding. Changes of inorganic anions in the extravesicular medium and of membrane potential by valinomycin altered the initial uptake activity of L-carnitine by OCTN2. In addition, the fluxes of L-carnitine and Na<sup>+</sup> were coupled with 1:1 stoichiometry. Accordingly, it was clarified that Na<sup>+</sup> is coupled with flux of L-carnitine and the flux is an electrogenic process. Furthermore, OCTN2 was localized on the apical membrane of renal tubular epithelial cells. These results clarified that OCTN2 is important for the concentrative reabsorption of L-carnitine after glomerular filtration in the kidney. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Membrane transport; Carnitine; OCTN2; Electrogenic transport; Sodium dependent transport; Membrane vesicle

### 1. Introduction

L-Carnitine (3-hydroxy-4-(trimethylammonio)butanoate) is a water-soluble vitamin and has a physiologically important role in the  $\beta$ -oxidation of long-chain fatty acids by facilitating transport of fatty acids across the inner membrane of mitochondria as acylcarnitines. L-Carnitine is supplied both from the diet and by biosynthesis in certain tissues such as liver, so it has to be delivered from plasma to the

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intracellular space of the tissues where  $\beta$ -oxidation proceeds. Furthermore, although L-carnitine is eliminated from the kidney by glomerular filtration, it is retained in the body by reabsorption in the renal epithelial cells. Accordingly, L-carnitine must be transported across cellular membranes in kidney and other tissues. There have been many studies on L-carnitine transport using animals and human tissue samples, and  $\text{Na}^+$ -dependent and -independent systems have been identified [1–5]. They are also classified as high-affinity and low-affinity transport mechanisms, and both types may function in parallel. The  $\text{Na}^+$ -dependent high-affinity transporter seems to be important for the concentrative uptake of L-carnitine into tissues from plasma, in which the physiological concentration is about 50  $\mu\text{M}$ . Systemic carnitine deficiency (SCD), which causes severe symptoms in childhood, such as cardiomyopathy, skeletal myopathy and hyperglycemia, is caused by an inherited functional defect of  $\text{Na}^+$ -dependent high-affinity carnitine transporter(s) in kidney and muscle, thereby decreasing reabsorption in kidney and distribution to muscle in humans [6–8].

In  $\text{Na}^+$ -dependent transport of L-carnitine, two mechanisms have been reported on the basis of various experimental methods, including  $\text{Na}^+$ -dependent binding and  $\text{Na}^+$ -dependent transport. In human placental brush-border membrane vesicles, apparent uptake of L-carnitine was significantly high in the presence of  $\text{Na}^+$ , although the apparent uptake did not show uphill transport, namely overshoot uptake, which is typically observed in the  $\text{Na}^+$ -coupled transport of substrates such as D-glucose [9]. In renal brush-border membrane vesicles, overshoot uptake was observed in the presence of an inwardly directed  $\text{Na}^+$  gradient [9–11], suggesting the presence of  $\text{Na}^+$ -coupled transport of L-carnitine. However, one of those studies showed  $\text{Na}^+$ -dependent but electroneutral transport of L-carnitine [10], while the other study demonstrated an enhancement of L-carnitine transport by inside-negative membrane potential [11]. So, at present the mechanism of the  $\text{Na}^+$  dependence observed in L-carnitine transport is not well established.

As one of the  $\text{Na}^+$ -dependent high-affinity transporters of L-carnitine, we isolated human and mouse OCTN2, which are members of the carnitine/organic cation transporter OCTN family [12–14]. The first

member of the OCTN family, OCTN1, showed structural similarity to members of the previously known organic cation transporter OCT family and exhibited sodium-independent organic cation transport activity [12,15,16]. OCTN2, isolated as a homologue of OCTN1, showed organic cation transport activity, as well as carnitine transport activity [17–19]. The  $K_m$  values of human OCTN2 for L-carnitine and the organic cation, tetraethylammonium (TEA), were 4.3  $\mu\text{M}$  and 304  $\mu\text{M}$ , respectively ([13] and unpublished observation). In addition, human OCTN2 showed predominant  $\text{Na}^+$  dependence in L-carnitine transport. Therefore, OCTN2 is the first  $\text{Na}^+$ -dependent high-affinity transporter of L-carnitine. The physiological importance of OCTN2 was first identified by the analysis of mutations in the OCTN2 gene of SCD patients and their families, as well as in a SCD model animal, *juvenile visceral steatosis (jvs)* mouse [20]. At present, various mutations of the OCTN2 gene in SCD patients are known and OCTN2 has been confirmed as the causative gene for SCD [21–25]. Since OCTN2 is expressed in many tissues, including kidney, placenta, skeletal muscle and heart, it is assumed that OCTN2 may be functional in these tissues, and previous findings of L-carnitine transport in these tissues may be ascribed at least in part to OCTN2. Thus, it is essential to clarify the mechanism of the  $\text{Na}^+$  dependence of L-carnitine transport via OCTN2, because the role of  $\text{Na}^+$  in L-carnitine transport is still controversial and the result may allow us to identify the responsible carnitine transporter molecule in each tissue.

The purpose of the present study is to clarify the role of  $\text{Na}^+$  in OCTN2-mediated L-carnitine transport. Sodium dependence has several mechanisms, such as effect on specific binding, specific coupling with substrate transport, and other allosteric effects. In the transport of nutrients such as D-glucose and amino acids, a sodium ion gradient supplies energy for uphill transport of substrates, and in such a case OCTN2 can accumulate L-carnitine against a concentration gradient. We therefore examined the possibility of  $\text{Na}^+$ -coupled transport of L-carnitine via human OCTN2 using membrane vesicles prepared from cultured cells transfected with human OCTN2 cDNA, since membrane vesicles are suitable for studies to identify the driving force of a transporter by manipulating intra- and extravesicular constituents.

## 2. Materials and methods

### 2.1. Reagents

L-[methyl-<sup>3</sup>H]Carnitine hydrochloride (65 Ci/mmol) was purchased from Moravec Biochemicals (Brea, CA, USA). Other reagents, including those for cell culture, membrane preparation and transport experiments, were obtained from Sigma Chemicals (St. Louis, MO, USA), Wako Pure Chemical Industries (Osaka, Japan), and Life Technologies (Rockville, MD, USA). HEK293 cells were obtained from Health Science Research Resources Bank (Tokyo, Japan).

### 2.2. Cell culture and preparation of plasma membrane vesicles

HEK293 cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Life Technologies), penicillin, and streptomycin in a humidified incubator at 37°C and 5% CO<sub>2</sub>. HEK293 cells were transfected with human OCTN2 subcloned into the *Bam*HI site of pcDNA3, an expression vector DNA (Invitrogen, San Diego, CA, USA), or pcDNA3 vector alone using LipofectAMINE as follows. Ten micrograms of pcDNA3 with or without human OCTN2 cDNA insert and 30 µl of Lipofectamine reagent (Life Technologies) were incubated at room temperature for 45 min in serum-free culture medium and were added to HEK293 cells that had been cultured and rinsed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline (pH 7.4). The next day the cells were plated at a density of 2 × 10<sup>5</sup> cells/dish (100 mm). The cells were cultured in the presence of 1 mg/ml G418 (Life Technologies) and a single colony was isolated at day 14. The expression of human OCTN2 was screened by measuring the uptake of L-[<sup>3</sup>H]carnitine and reverse transcription (RT)-PCR analysis as described below.

The method used for membrane vesicle preparation is essentially as described previously [26]. The obtained cell clone that expresses human OCTN2 was cultured on 30 plastic dishes (150 mm in diameter) containing 1 mg/ml G418 and when they reached confluence the cells were harvested by scraping with rubber policemen and washed twice by cen-

trifugation in phosphate-buffered saline (PBS). The cells were suspended in 25 ml of buffer 1 (10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.02 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl, pH 7.4) and placed on ice for 30 min. The cells were incubated in a cell disruption bomb (Parr 4635) and equilibrated at 700 psi by nitrogen gas with gentle stirring for 20 min. Then the pressure was released, ethylenediaminetetraacetic acid was added to a final concentration of 1 mM and the content was homogenized with a Teflon-glass homogenizer (20 strokes) at 4°C. The cell homogenate was collected and centrifuged for 5 min at 1000 rpm and 4°C. The supernatant was layered on buffer 2 (10 mM Tris-HCl, pH 7.4 and 35% sucrose) in a centrifuge tube and centrifuged for 90 min at 18 000 × *g* and 4°C. The white-colored fluffy layer at the boundary of buffer 2 was collected, suspended in buffer 3 (150 mM mannitol, 75 mM potassium gluconate and 10 mM HEPES-Tris, pH 7.4) and centrifuged at 100 000 × *g* for 2 h at 4°C. The resultant pellet was again suspended in buffer 3 and centrifuged at 100 000 × *g* for 3 h at 4°C. The final pellet was suspended in buffer 3 and stored at –80°C until used for transport experiments. The content of protein in each preparation was measured by the method reported by Bradford, using a Bio-Rad protein assay kit [27].

### 2.3. Transport measurement by membrane vesicles

Transport studies were performed by a rapid filtration technique at 25°C. Membrane vesicles stored at –80°C were thawed rapidly at 37°C and kept on ice. After 30 min preincubation at 25°C, 10 µl of membrane vesicle suspension containing 20 µg protein was mixed with 90 µl of drug solution containing radiolabeled L-carnitine in buffer 4 (usually 150 mM NaCl, 20 mM mannitol, 10 mM HEPES-Tris, pH 7.4) to initiate uptake studies. The uptake reaction was terminated by diluting with 1 ml of buffer 5 (20 mM mannitol, 160 mM potassium chloride, 5 mM HEPES-Tris, pH 7.4) at 4°C. The diluted sample was filtered immediately using a membrane filter (Millipore, HAWP, 0.45 µm pore size) and washed four times with buffer 5. The radioactivity retained on the membrane filter was quantitated with a liquid scintillation counter.

## 2.4. RT-PCR analysis

Total RNA from HEK293 cells was isolated by using Rneasy MINI kit (Qiagen). After treatment of total RNA with DNase I, first strand cDNA was prepared from 4.8 µg of RNA and amplified using OCTN2-specific primer pairs (5'-CATCTT-TGTGAAGCTGCTTCCTTTC-3' and 5'-TGACTCC-AAACTTGCCCACCATCA-3'). These primers correspond to nucleotide positions 1236–1259 (sense) and 1418–1441 (antisense) of OCTN2 cDNA. PCR was performed for 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

## 2.5. Data analysis

Initial uptake rates were obtained by measuring the uptake at 1 min. To estimate kinetic parameters for saturable transport of L-carnitine and the stoichiometry between Na<sup>+</sup> and L-carnitine, the uptake rates were fitted to the following equations by means of nonlinear least-squares regression analysis using WinNonlin (Scientific Consulting, Cary, NC).

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

$$v' = V_{\max} \times [\text{Na}^+]^n / \{K_m^n + [\text{Na}^+]^n\} \quad (2)$$

where  $v$  and  $v'$  and  $s$  are the uptake rates and concentration of substrate (L-carnitine or sodium ions), respectively, and  $K_m$ ,  $V_{\max}$  and  $n$  are the half-saturation concentration (Michaelis constant), the maximum transport rate and the Hill coefficient, respectively. All data were expressed as mean  $\pm$  S.E.M. and statistical analysis was performed by use of Student's  $t$ -test. The criterion of significance was taken to be  $P < 0.05$ . Each transport study was repeated at least three times and they gave essentially the same results, so typical results are shown here.

## 2.6. Immunohistochemical study of OCTN2 in kidney

Rabbit anti-OCTN2 polyclonal antibody was raised against a synthetic peptide corresponding to the COOH-terminal 19 amino acid residues of mouse OCTN2 (TRMQKDGEESPTVLKSTAF), as described previously [14]. The COOH-terminal of rat homologue (TRTQKDGGESPTVLKSTAF) differs from the mouse sequence in two amino acid residues,

T of the third and G of the eighth position, as shown in *italic* [28,29]. Immunohistochemical analysis was performed as described elsewhere [30] with minor modifications. In brief, after fixation of the mice and rats by perfusion with 4% paraformaldehyde, the kidneys were removed by dissection and further fixed by immersion in the same fixative. They were then frozen and cut into 10 µm sections by means of a cryostat. The sections were first incubated with anti-OCTN2 polyclonal antibody at 1/10 dilution or normal rabbit IgG (Dako) for 2 h at room temperature. The site of immunoreaction was revealed by successive treatment of the sections with biotinylated anti-rabbit IgG antibody, Avidin DH, biotinylated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA), and 3',3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries) in the presence of 0.006% hydrogen peroxide. The specimens were examined with an Axiovert S 100 microscope (Carl Zeiss) and the images were captured with an MC 80 DX camera.

## 3. Results

### 3.1. Establishment of HEK293 cells stably transfected with human OCTN2

Among HEK293 cell lines transfected with human OCTN2, one clone was selected as a stably OCTN2-expressing cell line. Fig. 1a shows the uptake activity of L-[<sup>3</sup>H]carnitine by the cell line, compared with the uptake by cells transfected with the expression vector pcDNA3 alone (mock). The uptake of L-[<sup>3</sup>H]carnitine by the OCTN2-transfected cell line finally selected was  $26.8 \pm 0.87$  pmol/mg protein/3 min at the concentration of 10 nM, which is about 173 times higher than that by the cell line transfected with expression vector pcDNA3 alone (mock,  $0.155 \pm 0.004$  pmol/mg protein/3 min). To confirm that the activity can be ascribed to OCTN2 gene transfection, expression of messenger RNA of OCTN2 was studied by RT-PCR. In human OCTN2-transfected cells the corresponding band to OCTN2 gene was observed and the band was not detectable in mock cells, while it cannot be excluded that OCTN2 is expressed in native HEK293 cells to some extent. From these results, the cell line was

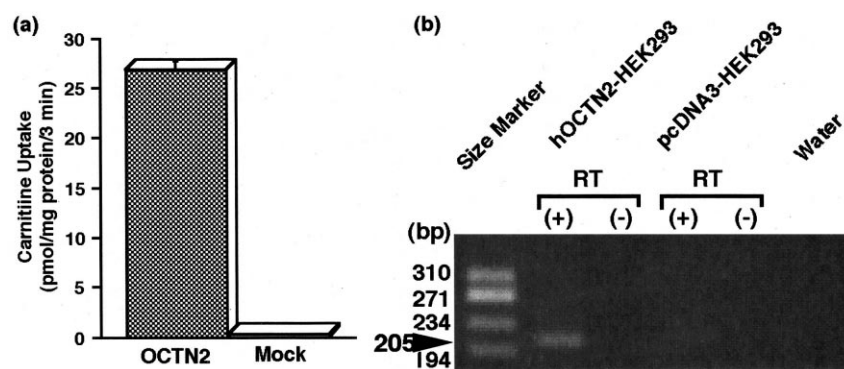


Fig. 1. Establishment of stably OCTN2-expressing HEK293 cells. a shows the uptake of L-[ $^3$ H]carnitine (10 nM) by HEK293 cells transfected with human OCTN2 (closed bar) or with vector pcDNA3 alone (open bar). b shows the expression of human OCTN2 mRNA detected by RT-PCR. The reaction was performed as described in Section 2 and the RT-PCR products were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. The specific product from human OCTN2 was 205 bp in size.

confirmed to be adequate to use for the characterization of OCTN2 function by correcting the uptake by mock cells from the apparent uptake by the OCTN2-expressing cells.

### 3.2. Time course and $\text{Na}^+$ dependence of L-carnitine uptake

Uptake of L-[ $^3$ H]carnitine by OCTN2 was measured using membrane vesicles prepared from HEK293 cells transfected with OCTN2 cDNA or

cells expressing pcDNA3 vector alone (mock cells). Fig. 2a shows the time courses of the uptake of L-[ $^3$ H]carnitine in the presence and absence of extravesicular  $\text{Na}^+$ . Here, in the absence of  $\text{Na}^+$ , the ions were replaced with *N*-methylglucamine. Overshoot uptake of L-[ $^3$ H]carnitine was observed only in the presence of  $\text{Na}^+$  by membrane vesicles from the OCTN2-expressing cells. No overshoot uptake in the presence of  $\text{Na}^+$  was observed in the case of membrane vesicles from mock cells. Although steady-state uptake by OCTN2-bearing membrane

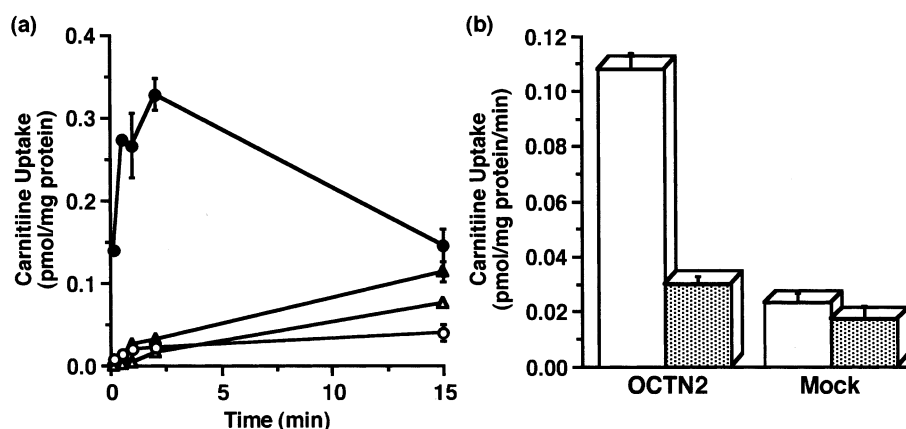


Fig. 2. Time course of L-carnitine uptake by membrane vesicles expressing OCTN2 (a) and osmolarity effect (b). Membrane vesicles from OCTN2-expressing (circles) and mock HEK293 cells (triangles) were suspended in buffer 3 containing 75 mM potassium gluconate, 150 mM mannitol, 10 mM HEPES–Tris, pH 7.4. Uptake of L-[ $^3$ H]carnitine (40 nM) was measured in the buffer containing 20 mM mannitol and 150 mM sodium chloride (closed symbols) or *N*-methylglucamine chloride (open symbols) at pH 7.4 buffered by 10 mM HEPES–Tris. In the study of the osmolarity effect on L-[ $^3$ H]carnitine uptake, uptake was terminated using isotonic (160 mM potassium chloride, 20 mM mannitol and 5 mM HEPES–Tris, pH 7.4, open bar) or hypotonic (5 mM HEPES–Tris, pH 7.4, dotted bar) stop solution. The osmolarity of stop solutions was about 320 mosm/kg or 5 mosm/kg, respectively. The results are shown as means and S.E.M. of four determinations. When the S.E.M. is smaller than the symbols, it is not shown.

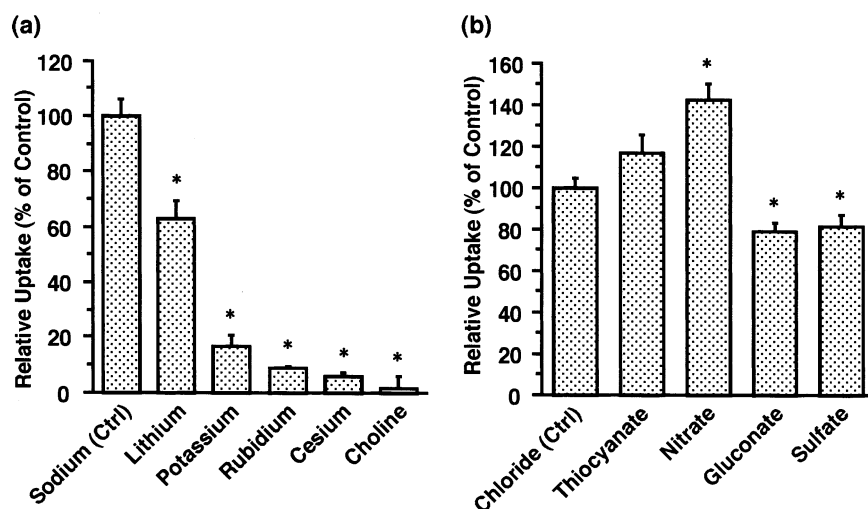


Fig. 3. Effect of inorganic cations and anions on OCTN2-mediated uptake of L-[<sup>3</sup>H]carnitine by membrane vesicles. Uptake of L-[<sup>3</sup>H]carnitine (40 nM) by OCTN2-expressing and mock vesicles was measured for 1 min. When sodium or chloride ions were replaced with other cations or anions, chloride or sodium ions were used as counterions, respectively. The results are shown as OCTN2-specific uptake, obtained by subtracting the uptakes by mock membrane vesicles from those by OCTN2-expressing vesicles, and expressed as a percentage of the uptake in the presence of sodium chloride. Each value represents the mean and S.E.M. of three determinations. \*Significantly different from control (sodium chloride buffer).

vesicles showed higher uptake in the presence of Na<sup>+</sup>, the difference was very small compared with the difference at the initial stage. These observations demonstrated that OCTN2 mediates the Na<sup>+</sup>-coupled transport of L-carnitine. Fig. 2b shows the osmolarity effect on the initial uptake of L-carnitine by the membrane vesicles. When membrane vesicles were collapsed by washing with low osmolarity medium, the apparent initial uptake was significantly decreased in the case of OCTN2-expressing membrane vesicles, while the osmolarity effect was minimum in membrane vesicles from mock cells. So, the observed overshoot transport of L-[<sup>3</sup>H]carnitine by OCTN2-expressing cells shown in Fig. 2a was largely accounted for by intravesicular accumulation rather than the binding to the membranes, although there is some contribution of Na<sup>+</sup>-dependent binding of L-carnitine to the membrane vesicles as reported in other studies [9].

### 3.3. Cation and anion dependence of OCTN2-mediated uptake

To study the specificity of L-carnitine uptake by OCTN2 to Na<sup>+</sup>, the effect of replacement of Na<sup>+</sup> in the extravesicular medium with various cations

was examined. Fig. 3 shows the initial uptake of L-[<sup>3</sup>H]carnitine by OCTN2-expressing and mock membrane vesicles. The results are shown as OCTN2-specific uptake after correction for the uptake by mock membrane vesicles. L-[<sup>3</sup>H]Carnitine uptake was highest in the presence of Na<sup>+</sup>, and lithium ions partially supported the uptake activity, while other cations such as potassium, rubidium, cesium or choline afforded negligible uptake. Here, choline showed the lowest uptake; this may be explained by competitive inhibition of L-carnitine uptake, because a high concentration of choline reduces the uptake of L-carnitine via OCTN2. The effect of inorganic anions was also studied by replacing chloride ions with thiocyanate, nitrate, gluconate or sulfate ions. Compared with chloride ions, thiocyanate and nitrate ions afforded higher uptake and gluconate and sulfate ions afforded lower uptake, although the effects were small.

### 3.4. Concentration dependence on L-carnitine uptake and stoichiometry with Na<sup>+</sup>

The concentration dependence of Na<sup>+</sup>-dependent L-carnitine uptake by OCTN2-expressing membrane vesicles was studied. The initial uptake rate was de-

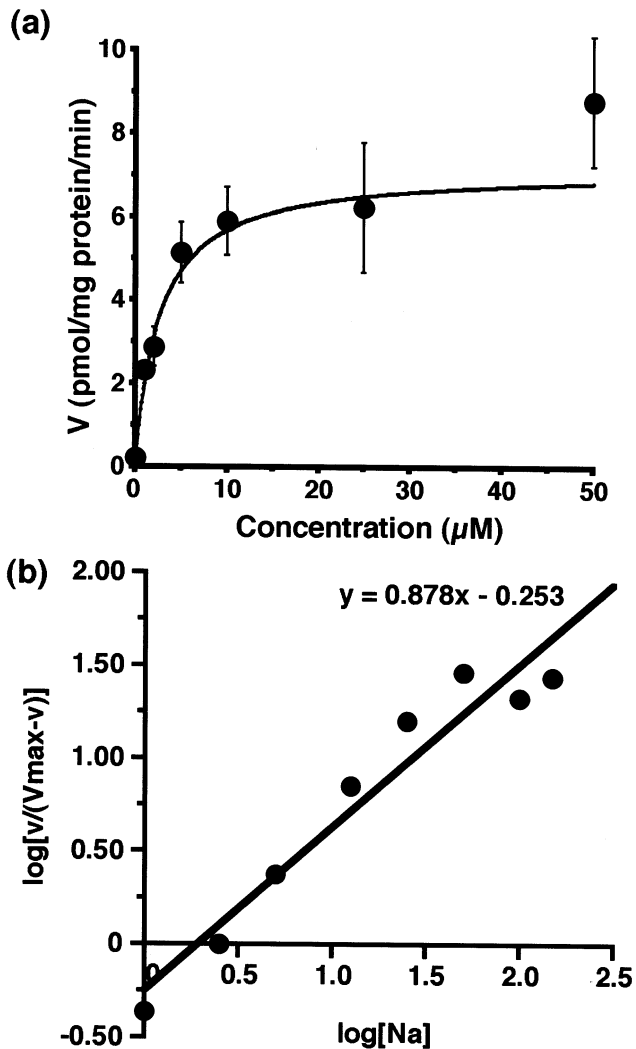


Fig. 4. L-Carnitine (a) and sodium ion (b) concentration dependences of OCTN2-mediated uptake of L-carnitine. (a) Initial uptake of L-carnitine by membrane vesicles at various concentrations of L-carnitine from 40 nM to 50  $\mu\text{M}$  was measured for 1 min. The uptakes by OCTN2 were obtained by subtracting those of membrane vesicles prepared from mock cells from the total. The solid line represents the uptake estimated from the kinetic parameters,  $K_m$  and  $V_{\max}$ , shown in Section 3. (b) L-[ $^3\text{H}$ ]Carnitine uptake (40 nM) by membrane vesicles was measured at various concentration of sodium ion from 0 to 150 mM for 1 min. OCTN2-mediated uptake was obtained as the uptakes by OCTN2-expressing vesicles minus those by mock vesicles and is shown by Hill plots. Sodium ions were replaced with *N*-methylglucamine to maintain the osmolarity. The results are shown as means and S.E.M. of four determinations. When the S.E.M. is smaller than the symbols, it is not shown.

terminated at 1 min. After subtraction of the uptake by mock vesicles, the uptake of L-carnitine was saturable (Fig. 4a) and nonlinear least-squares analysis showed half-saturation concentration and  $V_{\max}$  values of  $2.66 \pm 0.75 \mu\text{M}$  and  $7.13 \pm 0.71 \text{ pmol/min/mg protein}$ , respectively. Uptake of a tracer concentration (40 nM) of L-carnitine exhibited a nonlinear relationship with extravesicular  $\text{Na}^+$  concentration with apparent half-saturation  $\text{Na}^+$  concentration of  $2.34 \pm 0.22 \text{ mM}$  as shown in Fig. 4b. Hill plot analysis showed that the stoichiometry of transport of L-carnitine and  $\text{Na}^+$  was 0.88, suggesting that one  $\text{Na}^+$  molecule is associated with the transport of one molecule of L-carnitine. Thus, it appears that transport of L-carnitine is an electrogenic process.

### 3.5. Effect of membrane potential on OCTN2-mediated L-carnitine uptake

The electrogenicity of  $\text{Na}^+$ -coupled L-carnitine transport via OCTN2 was studied by examining the effect of valinomycin-induced potassium diffusion

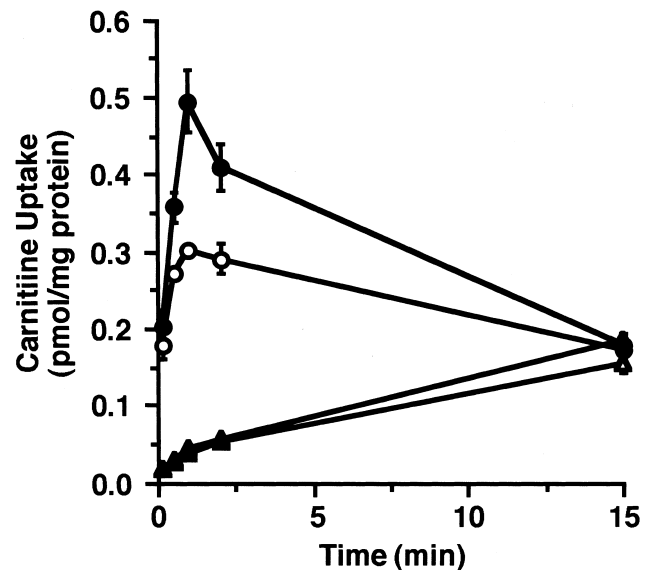


Fig. 5. Effect of valinomycin on OCTN2-mediated L-carnitine uptake by membrane vesicles. Uptake of L-[ $^3\text{H}$ ]carnitine (40 nM) was measured in 10 mM HEPES-Tris buffer, pH 7.4, containing 150 mM NaCl, 20 mM mannitol and 45  $\mu\text{M}$  valinomycin (closed symbols) or 0.25% ethanol alone (open symbols), which was used to dissolve valinomycin. Uptakes by OCTN2-expressing and mock vesicles are shown by circles and triangles, respectively. The results are shown as means and S.E.M. of four determinations. When the S.E.M. is smaller than the symbols, it is not shown.

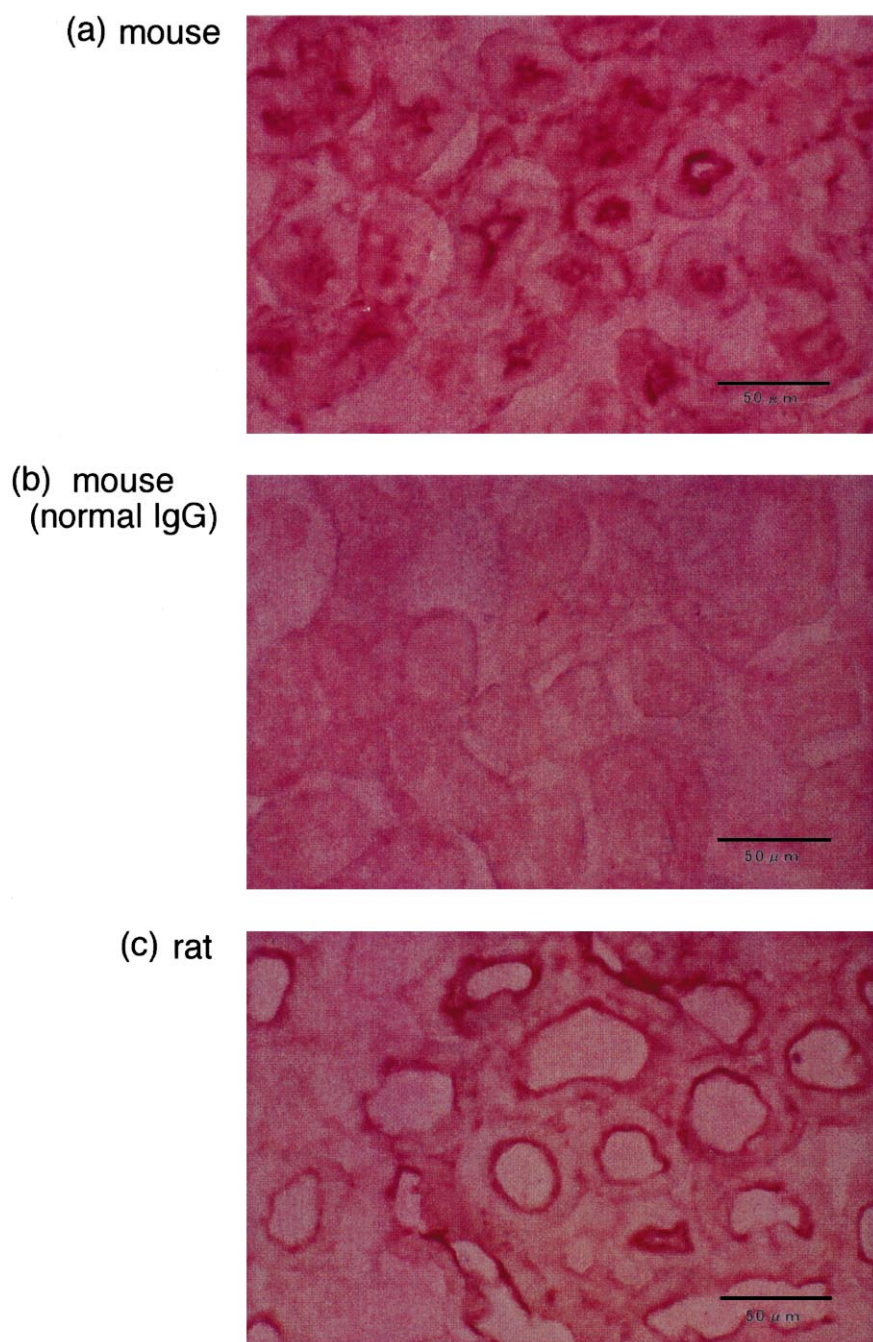


Fig. 6. Immunolocalization of OCTN2 protein in kidney of mice (a,b) and rats (c). Cryosections (10  $\mu$ m) of mouse and rat kidneys were incubated with the affinity-purified antiserum (10-fold dilution) against mouse OCTN2 (a,c) or normal anti-rabbit IgG (25  $\mu$ g IgG/ml) and examined under an Axiovert S 100 microscope. The apical membrane side of renal tubules was specifically stained by antibody against mouse OCTN2, in both mice and rats.

potential on L-carnitine uptake by the membrane vesicles in the presence of an outwardly directed potassium gradient, as shown in Fig. 5. When membrane potential was maintained relatively nega-

tive by using valinomycin, the initial uptake of L-[ $^3$ H]carnitine was transiently increased compared with that in the absence of valinomycin. In mock membrane vesicles no effect of valinomycin was ob-



served. Furthermore, the steady-state uptakes by membrane vesicles from OCTN2-expressing cells in the absence and presence of valinomycin were comparable. These results demonstrated that the effect of valinomycin cannot be ascribed to nonspecific damage to the membrane vesicles, and the alteration of L-[<sup>3</sup>H]carnitine uptake via OCTN2 is specific to the change of membrane potential. Considering this result, the effect of inorganic anions shown in Fig. 3b and the stoichiometry of L-carnitine and Na<sup>+</sup> in Fig. 4b demonstrate that Na<sup>+</sup>-dependent L-carnitine transport via OCTN2 is electrogenic.

### 3.6. Immunohistochemical localization of OCTN2 in kidney

OCTN2 is presumed to take up L-carnitine into renal tubular epithelial cells across the apical membrane in an Na<sup>+</sup>-dependent manner. So, the localization of OCTN2 in kidney was examined using anti-mouse OCTN2 antibody. We prepared an anti-peptide antibody to mice OCTN2. Since only two amino acid residues within the peptide sequence used for preparation of the antibody are different between mouse and rat OCTN2, the mouse OCTN2 antibody could be used for detection of OCTN2 in rat kidney. As shown in Fig. 6, the antibody reacted at the apical membrane of the renal epithelial cells (Fig. 6a,c), while no reaction was observed when normal IgG was used as a control (Fig. 6b). No staining was detected in basolateral membrane of the epithelial cells. So, OCTN2 was concluded to be expressed at the apical membrane of renal tubular epithelial cells.

## 4. Discussion

The present study was focused on the role of Na<sup>+</sup> in the OCTN2-mediated transport of L-carnitine. Previous studies on L-carnitine transport demonstrated that an Na<sup>+</sup>-dependent transport system is involved. However, some studies suggested that Na<sup>+</sup>-dependent binding of L-carnitine is predominant and L-carnitine transport is not necessarily coupled with the flux of Na<sup>+</sup> [4,5,9–11]. So, in the present study we examined whether Na<sup>+</sup> is transported with L-carnitine or not by studying L-carnitine up-

take by membrane vesicles prepared from cells transfected with human OCTN2.

First of all, we prepared HEK293 cells stably transfected with human OCTN2 by screening the L-carnitine transport activity and by RT-PCR specific to human OCTN2. Among several cell lines obtained, we chose one line that exhibited high expression of L-carnitine transport activity. The cell line was confirmed by RT-PCR to express the human OCTN2 gene. From this cell line we prepared membrane vesicles and further examined the transport of L-carnitine. To distinguish the binding of L-[<sup>3</sup>H]carnitine to the membrane vesicles from transport, the osmolarity effect on the uptake of L-[<sup>3</sup>H]carnitine was examined. The membrane vesicles were incubated with L-[<sup>3</sup>H]carnitine in the presence of extravesicular Na<sup>+</sup> for 1 min, for evaluation of the initial uptake of L-carnitine, then treated with standard isotonic or hypotonic stop solution to examine the effect of osmotic disruption. When membrane vesicles were disrupted by hypotonic stop solution, the uptake was significantly decreased in the case of the membrane vesicles from the OCTN2-transfected HEK293 cells, while there was little effect in the case of membrane vesicles from mock cells, demonstrating that the apparent uptake of L-[<sup>3</sup>H]carnitine in OCTN2-expressing vesicles was largely due to the intravesicular uptake rather than to the binding, although the partial contribution of Na<sup>+</sup>-dependent binding cannot be excluded as was demonstrated in other tissues [9]. In fact, when we evaluated the osmolarity effect on the steady-state uptake of L-carnitine to the membrane vesicles, it was estimated that about 10% of the total uptake was not sensitive to the extravesicular osmolarity (data not shown). Next, the time courses of uptake of L-[<sup>3</sup>H]carnitine were studied in the presence or absence of extravesicular Na<sup>+</sup> in OCTN2-expressing and mock membrane vesicles. The initial uptake of L-[<sup>3</sup>H]carnitine at 2 min by membrane vesicles from the OCTN2-expressing cells was very high in the presence of Na<sup>+</sup> and decreased by 15 min, showing a clear overshoot uptake. Uptakes of L-[<sup>3</sup>H]carnitine by OCTN2-expressing cells in the absence of Na<sup>+</sup> and by mock vesicles either in the presence or absence of Na<sup>+</sup> did not show such an increase at the initial state. Furthermore, the difference in the uptakes at the steady state between in the presence

and the absence of  $\text{Na}^+$  was small in OCTN2-expressing membrane vesicles. This result strongly suggests that  $\text{Na}^+$  is coupled with the transport of L-[ $^3\text{H}$ ]carnitine in a secondary active transport mechanism and that  $\text{Na}^+$ -dependent binding to OCTN2 is negligible in total uptake. When extravesicular  $\text{Na}^+$  was replaced with other cations, the initial uptake was significantly decreased, though lithium ions supported an intermediate uptake activity. Other cations such as potassium, rubidium, cesium, choline, or *N*-methylglucamine did not support the specific transport of L-[ $^3\text{H}$ ]carnitine. It is often observed that lithium ions partially support the  $\text{Na}^+$ -coupled transport. So, human OCTN2-mediated transport of L-carnitine has the characteristics of an  $\text{Na}^+$ -coupled mechanism. Partial stimulation of L-carnitine uptake by  $\text{Li}^+$  was also consistent with previous observations in  $\text{Na}^+$ -dependent transport of L-carnitine by the renal and placental brush-border membrane vesicles [9,11], while in the case of placenta, no overshoot uptake of L-carnitine was observed in the presence of  $\text{Na}^+$  gradient. Furthermore, hepatic basolateral membrane transport of L-carnitine also showed  $\text{Na}^+$  dependence and the activity was partially supported by  $\text{Li}^+$ . On the other hand,  $\text{Li}^+$  induced comparable uptake of L-carnitine with  $\text{Na}^+$  in rat muscle vesicles [5], suggesting an involvement of a transporter in muscle other than OCTN2. These results suggest that OCTN2 may be involved in the transport of L-carnitine in several tissues such as kidney, placenta and liver, but not in all tissues.

The initial uptake of L-carnitine was saturable with a  $K_m$  value of  $2.66 \pm 0.75 \mu\text{M}$ , which is consistent with the value obtained using HEK293 cells transiently transfected with human OCTN2,  $4.3 \mu\text{M}$  [14]. Based on the  $K_m$  value, the stoichiometry of L-carnitine and  $\text{Na}^+$  was examined by changing the extravesicular  $\text{Na}^+$  concentration from 0 to 150 mM at the L-[ $^3\text{H}$ ]carnitine concentration of 40 nM, which is significantly lower than the  $K_m$  value of L-carnitine itself or the sodium concentration used to allow the kinetic evaluation of the effect of  $\text{Na}^+$  concentration. The Hill coefficient 0.88 was close to unity as the same as observed in the study using HEK293 cells transiently expressing human OCTN2, 0.926 [18], whereas the apparent  $K_m$  value of  $\text{Na}^+$  ( $K_{m,\text{Na}^+}$ ) 2.34 mM in the present study was rather small compared with previously reported  $K_{m,\text{Na}^+}$  18.5 mM in

HEK293 cells [18]. The difference in  $K_{m,\text{Na}^+}$  may be ascribed to the differential experimental methods, but the Hill coefficient, which means the stoichiometry of the cotransport of L-carnitine and  $\text{Na}^+$ , was close to unity in both experiments. So, it is strongly suggested that one L-carnitine molecule is transported with one  $\text{Na}^+$  molecule via human OCTN2. Since L-carnitine is zwitterionic with the  $\text{pK}_a$  value of the carboxylic acid moiety being 3.8 [31] and a positively charged trimethylammonium moiety, it is assumed that the transport of L-carnitine with  $\text{Na}^+$  is electrogenic. When membrane potential was clamped by using valinomycin-induced potassium flux,  $\text{Na}^+$ -dependent uptake of L-[ $^3\text{H}$ ]carnitine was increased compared with the uptake in the absence of valinomycin. In addition, when the chloride ion in the extravesicular medium was replaced with other inorganic anions such as thiocyanate, nitrate, gluconate or sulfate ions, some changes in L-[ $^3\text{H}$ ]carnitine uptake were observed. Membrane-permeable nitrate significantly increased the uptake, while membrane-impermeable gluconate or sulfate decreased the uptake. Membrane-permeable thiocyanate showed some increase (not statistically significant). The effect of inorganic anions is not specific and may occur via a change of the membrane potential difference due to the difference of membrane permeability among ionic species. These observations suggest that membrane potential affects the transport of L-[ $^3\text{H}$ ]carnitine.

Overall, our results indicated that L-carnitine transport via OCTN2 is electrogenic owing to coupling with the flux of  $\text{Na}^+$ . Recently, it was demonstrated that L-carnitine transport is electrogenic in electrophysiological studies using *Xenopus* oocytes expressing human OCTN2 [32], although the flux of L-carnitine itself was not measured in that study. In the present study, we directly measured the electrogenic transport of L-carnitine itself and our finding is consistent with the result obtained by the electrophysiological approach. All of these results demonstrate that transport of L-carnitine via OCTN2 is coupled with  $\text{Na}^+$  and the effect of  $\text{Na}^+$  cannot be ascribed to binding to the transporter but to transport via OCTN2.

Physiologically, OCTN2 plays a role in the reabsorption of glomerularly filtered L-carnitine across the renal tubular epithelial cells, as well as in tissue distribution to liver and other tissues [33,34]. To

maintain the physiological concentration of L-carnitine, reabsorption in kidney is very important. An SCD animal model, *jvs* mouse, has mutated OCTN2, consequently lacking L-carnitine transport activity [20]. Plasma concentrations of free and total carnitine in *jvs* mice are significantly lower than those of wild mice and this is assumed to be caused by the absence of reabsorption in kidney, because renal clearance of L-carnitine is significantly increased in *jvs* mice [33]. So, we confirmed the localization of OCTN2 in kidney. In mice, OCTN2 is located on the luminal side of renal tubular epithelial cells according to immunohistochemical analysis. Similarly, the rat OCTN2 was found to be localized on the luminal side of the epithelial cells. In addition, our preliminary result of fluorescence immunohistochemical study suggested the limited expression of OCTN2 in proximal tubule but not in distal tubule of kidney. Therefore, we have demonstrated that OCTN2 is localized at the apical membrane of renal tubular epithelial cells and functions to reabsorb L-carnitine in kidney. Recently, we demonstrated that mouse OCTN2 exhibited a mutual trans-stimulation of Na<sup>+</sup>-dependent carnitine influx and an efflux of organic cation tetraethylammonium, suggesting an efficient transport for reabsorption of physiological compounds and secretion of xenobiotics in kidney [19]. However, in the absence of Na<sup>+</sup>, tetraethylammonium in the trans-side could not drive the influx of L-carnitine in HEK293 cells [19]. So, OCTN2 obligatory requires Na<sup>+</sup> for the active transport of L-carnitine and the organic cations in the trans-side may have only an additive effect on Na<sup>+</sup>-dependent L-carnitine transport via OCTN2.

In conclusion, it was demonstrated, using membrane vesicles prepared from cells transfected with OCTN2, that the Na<sup>+</sup> dependence of OCTN2-mediated transport of L-carnitine is due to coupled transport leading to the electrogenic, secondary active transport, but not to binding to the transporter. Furthermore, OCTN2 was shown to be located on the apical membrane of renal tubular epithelial cells. Although the roles of OCTN2 in other tissues are not yet known, it is already clear that OCTN2 is important to maintain L-carnitine concentration in the body by reabsorbing L-carnitine in a sodium-coupled electrogenic transport manner.

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