Molecular and Functional Characterization of Organic Cation/Carnitine Transporter Family in Mice*

Ikumi Tamiya, Rikiya Ohashi, Jun-ichi Nezu, Yoshimichi Sai, Daisuke Kobayashi, Asuka Oku, Miyuki Shimane, and Akira Tsuji

From the Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920-0934, Japan Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corp. (JST), Kawaguchi 332-0012, and Chugai Research Institute for Molecular Medicine Inc., Ibaraki 300-4101, Japan

Carnitine is essential for β-oxidation of fatty acids, and a defect of cell membrane transport of carnitine leads to fatal systemic carnitine deficiency. We have already shown that a defect of the organic cation/carnitine transporter OCTN2 is a primary cause of systemic carnitine deficiency. In the present study, we further isolated and characterized new members of the OCTN family, OCTN1 and -3, in mice. All three members were expressed commonly in kidney, and OCTN1 and -2 were also expressed in various tissues, whereas OCTN3 was characterized by predominant expression in testis. When their cDNAs were transfected into HEK293 cells, the cells exhibited transport activity for carnitine and/or the organic cation tetraethylammonium (TEA). Carnitine transport by OCTN1 and OCTN2 was Na+-dependent, whereas that by OCTN3 was Na+-independent. TEA was transported by OCTN1 and OCTN2 but not by OCTN3. The relative uptake activity ratios of carnitine to TEA were 1.78, 11.3, and 746 for OCTN1, -2, and -3, respectively, suggesting high specificity of OCTN3 for carnitine as well as carnitine itself.

Carnitine transport system that reabsorbs more than 95% of carnitine from the renal glomerular filtrate (2, 4–6). In 1988, mutant mice that showed a systemic carnitine deficiency (SCD) phenotype were found by Koizumi et al. (7) and were named juvenile visceral steatosis (jvs) mice. jvs mice exhibit fatty liver, hyperammonemia, and hypoglycemia. These symptoms are the phenotype of systemic carnitine deficiency in man (3, 8), and it was considered that a defect of the active transport system for carnitine may be related to SCD in human and mouse (3, 7). Wu et al. (11) and Tama et al. (9, 10) have isolated and characterized OCTN transporters OCTN1 and OCTN2 in human (9–11). The latter was shown to be a physiologically essential high-affinity Na+-dependent carnitine transporter by the demonstration that mutations found in SCD patients and jvs mice cause functional alteration of OCTN2 (9, 12). Other similar mutations in OCTN2 related to SCD have been reported (13–20). Interestingly, OCTN1 and -2 in human transported organic cations such as tetraethylammonium (TEA) as well as acylcarnitines (11, 21–23), suggesting that OCTNs may be important for the transport of xenobiotics and acylcarnitine as well as carnitine itself.

By means of an in vivo disposition kinetic study of carnitine, we demonstrated that the carnitine transporters are absent or functionally deficient in jvs mice because the renal reabsorption, the intestinal absorption, and the distribution to various tissues in these mice are significantly lower than those in wild-type mice (24). However, although functional loss of OCTN2 by mutation was found in jvs mice, the supplementation of carnitine or acetylcarnitine to jvs mice as well as patients improved their pathological symptoms (25–27). Furthermore, although the tissue distribution of carnitine was decreased in jvs mice, the observed tissue-to-plasma concentration ratios of carnitine in jvs mice are unity or above unity in several tissues (24). Because carnitine is soluble in water and is unlikely to be transported across the cell membrane via passive diffusion, it was strongly suggested that carnitine is concentrated in several tissues of jvs mice by active transport via a transporter other than OCTN2. We preliminarily demonstrated that carnitine was indeed transported by human OCTN1 in addition to OCTN2 (21). Accordingly, it is possible that additional carnitine transporters, which can partially compensate for loss of OCTN2 function, are present in human. Since carnitine metabolism is critical, it is important to clarify the transporters involved in carnitine disposition to understand the physiological roles of carnitine and to identify the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AB016257 and AB018438.

To whom correspondence should be addressed: Dept. of Pharmacodynamics, Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan. Tel.: 81-76-234-4479; Fax: 81-76-234-4477; E-mail: tsuji@kenroku.kanazawa-u.ac.jp.

1 The abbreviations used are: SCD, systemic carnitine deficiency; jvs, juvenile visceral steatosis; TEA, tetraethylammonium; RT, reverse transcription; PCR, polymerase chain reaction; OCT organic cation transporter; OAT, organic anion transporter.
cause of systemic and/or secondary carnitine deficiency. In the present study, we isolated new members of the mouse OCTN transporter family, mouse OCTN1 and OCTN3, and compared their functional characteristics as carnitine and/or organic cation transporters with those of OCTN2.

**EXPERIMENTAL PROCEDURES**

**Materials—**methyl-[^3]HAcetly-l-carnitine hydrochloride (65 Ci/ mmol) and l-methyl-[^3]Hcarnitine hydrochloride (85 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA). [1-[^3]C]-Tetraethylammonium bromide (2.4 mM/cnmol) was from NEN Life Science Products (Boston, MA). Other reagents were obtained from Sigma-Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), or Funakoshi Co. (Tokyo, Japan). HEK293 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan).

**Cloning of OCTNs cDNA and Tissue Distribution Study—**Mouse OCTN2 was cloned as described previously (12). OCTN1 and OCTN3 were cloned as follows. For OCTN1, a data base search using human OCTN1 cDNA sequence as the query identified several mouse Expressed Sequence Tags that show high homology to the query sequence. From these sequences, MONL 1 primer (5'-CGCCGCAATGCTGAAATTTCTTCGTC-3') and MONA 4 primer (5'-AGGCTTTGATTGTGGTCTGT-TGAGAC-3') were designed and used for PCR. A cDNA fragment of around 2 kilobase pairs was amplified by PCR from mouse kidney-derived cDNA. This fragment was sequenced and confirmed to have the full open reading frame of OCTN1. For OCTN3, in the course of cloning of OCTN2 by 5'-RACE (rapid amplification of cDNA ends), we found that a cDNA fragment that was highly homologous to OCTN2, but not identical with either OCTN2 or OCTN1, was co-amplified. As this fragment was considered to be derived from an unidentified OCTN1- and OCTN2-related gene, we termed it OCTN3 and cloned its full open reading frame. MONR 2 primer (5'-CGCCCCACTGGCUGCAGATATCCTC-3') and MONB 16 primer (5'-ATAGGCGAGGTTGATTCACAACTTT-3') were designed from the sequences of this fragment and mouse OCTN2, respectively. Using these primers a cDNA fragment of extended OCTN3 sequence was amplified by PCR from mouse embryo (17 days)-derived Marathon-ReadyTM cDNA (CLONTECH, Palo Alto, CA). Then MONC1 primer (5'-TGTCCTCTACGACCTACATTGT-3') and MONB 26 primer (5'-ACAGAACAGAAATGCGCGAGTGATCC-3') was designed from the newly obtained sequence and used to clone the 3' end of OCTN3 by 3'-RACE (rapid amplification of cDNA ends) from mouse testis-derived Marathon-ReadyTM cDNA (CLONTech). The whole sequence of OCTN3 was determined by assembling these sequences. cDNAs encoding the full open reading frame of OCTN1 and OCTN2-related genes were subcloned into the BamHI site of pcDNA3 vector (Invitrogen, San Diego, CA). Sequence-verified clones were selected and used for expression experiments.

**Tissue Distribution Study by RT-PCR and Western Blot Analyses—**In RT-PCR analysis, following member-specific primers were used for RT-PCR. OCTN1: MONL 1 primer (described above) and MONA 4 primer (described above). OCTN2: TCTTTCTCCGGGTTGCTGTGATGC-3') and MONB 26 primer (5'-ACAGAACGAAATGCGCGAGTGATCC-3'). OCTN3: MONR 2 primer (described above) and MONB 16 (described above). Using appropriate amounts of cDNA of a multiple tissue cDNA (MTCTM) panel (CLONTECH) derived from various mouse tissues and whole embryo as templates, each gene was amplified, and the expression levels were evaluated. For Western blot analysis, rabbit polyclonal antibodies were raised against synthesized polypeptides of the carboxyl terminus of mouse OCTN1, -2, and -3. Amino acid sequences used for OCTN1, -2, and -3 were NH2-CGKKST-VSVDREESPKVTL-COOH, NH2-CRMRQKDKESPTVLKSTAF-C-OOH, and NH2-CCKSKGNVSRSTKSEPFG-P-COOH, respectively. Mouse tissues were isolated and homogenized in 2 ml of buffer containing 210 mM sucrose, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 40 mM NaCl, 30 mM Hepes, 5 mM EDTA, 1 mM phenylmethylsulfonil fluoride, 1 μM aprotinin, 100 μM leupeptin, and 2 μg/ml aprotinin (pH 7.4) using Polytron homogenizer. Then 800 μl of the homogenate was mixed with 750 μl of 1.17 M KCl solution containing 58.3 mM tetrasodium pyrophosphate and centrifuged at 230,000 g for 75 min. The resultant pellet was suspended in 10 mM Tris-HCl and 1 mM EDTA (pH 7.4) centrifuged at 230,000 g again. The obtained pellet was suspended again in 600 μl of 10 mM Tris-HCl and 1 mM EDTA (pH 7.4) and dispersed ultrasonically. After the addition of 200 μl of 16% SDS solution, the solution was centrifuged at 15,000 g, and the resultant supernatant was used for Western blot analysis. The sample was separated by 12% SDS-polyacrylamide gel, proteins were transferred to polyvinylidene difluoride membrane Immobilon (Millipore, Bedford, MA), and the membrane was incubated in buffer, 20 mM Tris, 137 mM NaCl, 0.1% Tween 20 (pH 7.5) containing 10% skim milk. The membrane was incubated with respective polyclonal anti-peptide antibodies for 1 h. After washing with Tris buffer without skim milk three times, and incubated with secondary antibody (1:1000; Donkey anti-rabbit IgG, horseradish peroxidase-linked whole antibody (Amersham Pharmacia Biotech). The membrane was washed with the above buffer without skim milk, and the proteins were detected by enhanced chemiluminescence detection method using ECL Plus Western-blotting detection system (Amersham Pharmacia Biotech). Cultured cells transfected with OCTN1, -2, or -3 were obtained as described above, harvested, and treated as the same as for the tissue distribution experiments.

**Transport Study in HEK293 Cells—**The full-length OCTN cDNAs were subcloned into the BamHI sites of the expression vector pcDNA3 (Invitrogen, San Diego, CA), and the constructs, pcDNA3/OCTNs, were used to transfect HEK293 cells by means of the calcium phosphate precipitation method as described previously (10). HEK293 cells were cultured transfected with pcDNA3/OCTNs or pcDNA3 vector alone by adding 10 μg of the plasmid DNA per dish. 48 h after transfection, the cells were harvested and suspended in transport medium containing 125 mM NaCl, 4.5 mM KCl, 5.6 mM glucose, 1.25 mM KH2PO4, and 25 mM HEPES (pH 7.4). This suspension and a solution of a radiolabeled test compound in the transport medium were separately incubated at 37°C for 10 min, then transport was initiated by mixing them. At appropriate times, 200-μl aliquots of the mixture were withdrawn, and the cells were separated from the transport medium by centrifugal filtration through a layer of a mixture of silicon oil and liquid paraffin with a density of 1.03. Each cell pellet was solubilized in 3× KOH, then neutralized with HCl, and the associated radioactivity was measured by means of a liquid scintillation counter. HEK293 cells transfected with pcDNA3 vector alone were used to determine the background activity and are designated as Mock cells. Cellular protein content was determined according to the method of Bradford using a protein assay kit (Bio-Rad) and bovine serum albumin as the standard (28). In sodium-free experiments, sodium ions were replaced with N-methylglucamine, and the cells obtained were suspended in sodium-free medium. When transport was measured at acidic or alkaline pH (5.5–8.4), the pH was adjusted appropriately using HCl, NaOH, or KOH.

**Data Analysis—**Initial uptake rates were usually obtained by measuring the uptake at 3 or 30 min for carnitine and TEA, respectively. The saturation curves were expressed as the cell-to-medium concentration (CM) ratio (μmol of protein/5 min or 30 min), obtained by dividing the uptake amount in the cells by the concentration of test compound in the medium. To estimate kinetic parameters for saturable transport of carnitine or TEA, the uptake rate was fitted to the following equation by means of nonlinear least squares regression analysis using WinNonlin (Pharsight, Mountain View, CA).

$$ v = \frac{V_{\max} s}{K_m + s} $$

where $v$ and $s$ are the uptake rate and concentration of carnitine or TEA, respectively, and $K_m$ and $V_{\max}$ are the half-saturation concentration (Michaelis constant) and the maximum transport rate, respectively. All data were expressed as mean ± S.E., 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 by differently transfected cells, and they essentially showed the same results, so the typical results are shown.

**RESULTS**

**Amino Acid Sequences of Mouse OCTNs—**We isolated two new members of the mouse OCTN transporter family, OCTN1 and OCTN3 (Fig. 1a). The sequences of OCTN1 and -3 have been deposited in GenBankTM (accession numbers AB016257 and AB018436, respectively). The full-length OCTN1, OCTN2, and OCTN3 cDNAs appeared to encode polypeptides of 553, 557, and 564 amino acids and have 73, 86, and 78% similarities with human OCTN2 (9), respectively. The similarities with human OCTN1 were 84, 73, and 67%, respectively (10). Based on the similarities with human OCTN members, we designated the mouse OCTNs as shown in Fig. 1a. OCTNs exhibit about 30% similarity with organic cation transporters OCTs (29–34) and organic anion transporters OATs.
The phylogenetic tree of these transporters is shown in Fig. 1b, and it appears that the OCTNs form a distinct family of organic ion transporters.

**Tissue Distribution of OCTNs**—To compare the tissue distributions of OCTN1, -2, and -3, we performed RT-PCR and Western blot analysis in mouse tissues. Figs. 2, a and b, show the tissue distribution of OCTNs determined by RT-PCR and Western blot analyses, respectively. In RT-PCR analysis of adult mouse tissues, OCTN1 and OCTN2 were clearly expressed in kidney, liver, and testis and weakly in other tissues. They were also found in embryo. On the other hand, OCTN3 was strongly expressed in testis and weakly in kidney but was hardly expressed in other tissues. Antibodies were raised against the divergent COOH-terminal portions of each protein. The polyclonal antibodies were purified by affinity chromatography and were specific for OCTN1, -2, and -3, respectively, with no cross-reaction detectable on immunoblots as shown by the immunodetection of the cell lysates of HEK293 cells transfected with mouse OCTN1, -2, or -3 (Fig. 2b, left four lanes). Furthermore, HEK293 cells expressed all of OCTN1, -2, and -3 in significant amounts adequate for functional analysis in the following transport experiments. All antibodies recognized proteins of 64, 70, and 82 kDa for OCTN1, 70 and 80 kDa for OCTN2, and 54 and 60 kDa for OCTN3 in immunoblot of membranes from various mouse tissues. The estimated size of OCTN1, -2, and -3 from the deduced amino acid sequences were 62,287, 62,776,
and 63,317, respectively. There are variations in molecular size in the detected bands, and they are close to the estimated sizes. This is presumably ascribed to the variation in post-translational modification, degradation, or glycosylation. Expression profile of OCTN2 protein was broad, and strong expression was observed in kidney. OCTN1 protein was also detected in several tissues, and kidney showed strong expression. OCTN3 was characterized by strong expression in testis and weak expression in kidney without expression in other tissues. Therefore, most of the tissue expression profiles were comparable between OCTN genes and their products.

Functional Characterization of OCTNs Expressed in HEK293 Cells—Since human OCTN1 and OCTN2 transported TEA in a Na+-independent manner and carnitine in a Na+-dependent manner (9–11, 22), we studied the transport characteristics of mouse OCTN family members for both of carnitine and TEA by transient expression in HEK293 cells (Figs. 3, a and b). Uptakes of [3H]carnitine were increased linearly up to 10 min in OCTN2- and OCTN3-transfected HEK293 cells. In OCTN1-transfected cells, [3H]carnitine uptake was very slight, but the uptake activity expressed was statistically higher than that by Mock-transfected cells after...
The uptake of L-[^3H]carnitine and [^14C]TEA were measured for 10 min at 37 °C in transport buffer (pH 7.4) including sodium ion. The data were obtained by subtraction of the uptake by Mock-transfected cells from that by OCTN1-, OCTN2-, or OCTN3-transfected cells. Each value represents the mean ± S.E. of three determinations.

| Ratio of OCTNs | Uptake coefficient (µmol/g protein/10 min) | Relative uptake (carnitine/TEA) |
|---------------|---------------------------------------------|----------------------------------|
| L-Carnitine (10 nM) | TEA (100 µM) |                        |
| OCTN1         | 14.6 ± 0.5a | 8.2 ± 0.3a | 1.78          |
| OCTN2         | 117 ± 0.1b  | 10.3 ± 0.1b | 11.3         |
| OCTN3         | 74.6 ± 0.5b | 0.1 ± 0.1  | 746<         |

a Significantly different from the Mock-transfected cells by Student’s t test (p < 0.05).

FIG. 4. Na⁺ dependence of carnitine uptake by OCTN-expressing HEK293 cells. The uptake of L[^3H]carnitine (10 nM) was measured for 3 min at 37 °C in transport buffer (pH 7.4) in the presence (open columns) or absence (closed columns) of Na⁺. In the absence of Na⁺, Na⁻ was replaced with N-methylglucamine. The results show the values after subtracting the uptake by Mock cells from that by the OCTN-transfected cells. The results are shown as means ± S.E. of three determinations. The asterisks (*) indicates a significant difference from the presence of Na⁺ in the transport medium.

10 min. Because the same results were obtained by differently transfected cells, OCTN1 should have low, but existing, carnitine transport activity. Uptake of [^14C]TEA was increased up to 30 min by OCTN1- and OCTN2-transfected cells, whereas its uptake by OCTN3-transfected cells was negligible over 60 min. Comparison of relative transport activities of three OCTNs by correcting the expressed protein amount obtained in Western blot analysis was difficult due to the variation of titer among antibodies, although they recognize each protein specifically (Fig. 2b). Therefore, to avoid the difference of the expression level of respective protein in cells and to allow comparison of the substrate preference among the three members, the relative transport activities for carnitine and TEA were evaluated as summarized in Table I. Here, because OCTN3 essentially is unlikely to transport TEA, we used the apparently expressed transport activity as the difference between OCTN3-transfected and Mock cells. The ratios obtained by dividing the uptake of [^3H]carnitine (10 nM) by that of [^14C]TEA (100 µM) at 10 min were 1.78, 11.3, and >746 for OCTN1, -2, and -3, respectively. The result suggests that OCTN3 is highly specific for carnitine, OCTN1 has a preference for TEA, and OCTN2 has intermediate specificity.

Fig. 4 shows the Na⁺ dependence of OCTNs-mediated [^3H]carnitine uptake. OCTN1- and OCTN2-mediated [^3H]carnitine uptakes were much greater in NaCl-containing medium than in N-methyl-D-glucamine chloride-containing medium (i.e. in the absence of Na⁺). However, OCTN3-mediated [^3H]carnitine uptake was not significantly different between the presence and the absence of Na⁺. So, OCTN3 seems to have very distinct functional properties from the other OCTNs. In addition, the pH dependence of OCTN2- and OCTN3-mediated [^3H]carnitine uptake is shown in Figs. 5, a and b. When the pH in the transport medium was acidic, pH 5.5 and 6.0, OCTN2-mediated [^3H]carnitine uptake was significantly decreased to approximately 40 and 70% that at pH 7.4, respectively (p < 0.05). At alkaline pH 8.4, [^3H]carnitine uptake via OCTN2 was increased to approximately 120% that at pH 7.4 (p < 0.05), whereas the uptake of [^3H]carnitine by OCTN3- or Mock-transfected cells was scarcely affected by pH.

The concentration dependence in the transport of carnitine and TEA via OCTNs were examined to estimate the affinity. Uptakes of carnitine and TEA by OCTNs were saturable, and the Eadie-Hofstee plot showed a single straight line in each case, demonstrating the presence of a single functional site on the OCTN proteins (Figs. 6 and 7). The estimated Kₘ values of carnitine uptake via OCTN2 and OCTN3 were 22.1 ± 1.11 and 2.99 ± 0.49 µM, respectively, and the Vₘₜₐₓ values were 1.48 ± 0.03 and 0.16 ± 0.01 nmol/mg of protein/3 min, respectively. The Kₘ value of TEA uptake via OCTN1 and OCTN2 were 452 ± 41.0 and 215.7 ± 22.7 µM, respectively, and the Vₘₜₐₓ values were 11.2 ± 0.75 and 6.02 ± 0.25 nmol/mg of protein/30 min, respectively. The kinetic parameters of carnitine uptake by OCTN1 and TEA uptake by OCTN3 could not be determined because of the difficulty in analyzing them due to the low or negligible transport activity.

Substrate specificity of OCTN2- and OCTN3-mediated carnitine uptake was examined in terms of the inhibitory effect on the initial uptake of [^3H]carnitine (Table II). Uptakes of [^3H]carnitine by OCTN2 and OCTN3 were significantly inhibited by unlabeled carnitine (5 µM), acylcarnitines (5 µM), betaines (5, 50 or 500 µM), and TEA (500 µM). In the case of OCTN2, choline (500 µM), an endogenous organic cation, reduced the uptake of [^3H]carnitine. However, lysine or γ-aminobutyrate was not inhibitory. In the case of OCTN3, stronger inhibitory effects of L-carnitine and acylcarnitines were observed compared with the case of OCTN2, whereas glycinobetaine, choline, and TEA showed weaker inhibitory effects. These results suggest that OCTN3 has a higher specificity than OCTN2 for carnitine and related compounds, or in other words, OCTN2 has broader range of specificity than does OCTN3.

DISCUSSION

In the present study, we isolated two new members of the carnitine/organic cation transporter OCTN family in mouse, mouse OCTN1 and -3 (Fig. 1a), and compared their tissue distributions and functional properties with those of the previously reported OCTN2 to characterize them and to elucidate their physiological functions.

As shown in the phylogenetic tree in Fig. 1b, mouse OCTNs seem to form an organic ion transporter family distinct from the organic cation (OCT) and organic anion (OAT) transporter families. By the RT-PCR method, OCTN1 and OCTN2 were widely expressed in all of adult tissues examined in variable amounts, whereas OCTN3 was expressed only in testis strongly and weakly in kidney (Fig. 2a). OCTN2 has been determined to be directly related to SCD (12), and in the
present study it was confirmed by the strong expression of the protein in various tissues by RT-PCR and Western blot analysis (Fig. 2b). Especially, strong expression of OCTN2 protein in kidney demonstrated its contribution to the reabsorption of carnitine from urine after glomerular filtration to maintain carnitine content in body. OCTN1 protein was also detected strongly in kidney, whereas the expression of OCTN3 was very low. These tissue distribution profiles were rather comparable between expressions of the genes and their products. OCTN1 and -2 were continuously expressed in embryonic tissues from 7 to 17 days after birth, whereas OCTN3 was detected only in 7-day embryo. Thus, the tissue distributions of OCTN family members are different, and the strong expression of OCTN3 in testis and limited expression in embryo may reflect a specific role different from those of other members, as discussed below. Skeletal muscle and heart accumulate carnitine. RT-PCR showed low expression of both OCTN1 and -2 in heart, and they were also detected strongly by Western blot analysis. Although by RT-PCR OCTN1 and -2 were expressed in skeletal muscle, we failed to detect the signals of them by Western blot analysis (data not shown). Recently, similar but differential characteristics of carnitine transport from OCTN2 was reported by using isolated membrane vesicles in rat skeletal muscle, suggesting that OCTN1 or others distinct from OCTN2 participate in muscle in rats (40). Therefore, since human OCTN2 is strongly expressed in skeletal muscle (9), mouse and rat may have different regulatory mechanisms of carnitine disposition from human in terms of carnitine transport in skeletal muscle.

When the OCTNs were expressed in HEK293 cells, significantly increased uptake of carnitine was observed with all three OCTNs, although the expressed activity was very small in the case of OCTN1 (Fig. 3). Rat OCTN1 hardly transports
transport was also high affinity (4.34 μM) (9), so OCTN2 appears to be the major Na⁺-dependent carnitine transporter common to all these species. Uptake activity for TEA was observed in mouse OCTN1- and OCTN2-expressing cells, whereas TEA transport by OCTN3 was negligible (Fig. 3). In addition, $K_m$ of OCTN3 for carnitine was smaller (2.99 μM) than that of OCTN2 (22.1 μM), and the effects of inhibitors on OCTN3 were specific compared with OCTN2, showing a higher specificity of mouse OCTN3 for carnitine. Furthermore, OCTN1 and OCTN2 exhibited Na⁺-dependent carnitine uptake, whereas OCTN3 did not show Na⁺ dependence (Fig. 4). These observations strongly imply that mouse OCTN3 is a different type of carnitine transporter from OCTN1 or -2, showing higher specificity for carnitine, Na⁺ independence, and a narrow tissue distribution. Transport of TEA via mouse OCTN1 and OCTN2 exhibited lower affinity, with $K_m$ values of 452.3 and 215.7 μM, respectively. Thus, mouse OCTN1 and -2 accept both carnitine and TEA as substrates but have higher affinity for carnitine than for the organic cation TEA. Interestingly, mouse OCTN1 and -2 transport carnitine in a Na⁺-dependent manner, whereas TEA transport by them was Na⁺-independent. Accordingly, mouse OCTN1 and OCTN2 appear to be multispecific transporters, mediating organic cation transport as well as carnitine transport by different mechanisms, as has been demonstrated in the human counterparts (9–11, 21–23).

Detection of OCTN1 gene in liver is in contrast with the absence of human OCTN1 in liver (10), demonstrating a species difference. Cultured human hepatoma HLF cells expressed OCTN2 strongly and OCTN1 slightly (43). In in vitro studies of carnitine uptake by hepatocytes isolated from jvs mice, which genetically lack OCTN2-mediated carnitine transport, a significant decrease of accumulation of carnitine compared with that by wild-type mice was seen (44). Furthermore, when we studied in vivo carnitine disposition in jvs mice, the accumulation of carnitine (41), and the carnitine transport activity of human OCTN1 was significantly lower than that of human OCTN2 (21). In addition, the specificity of carnitine transport of mouse OCTN1 in relation to the organic cation TEA was the lowest among the three members (Table I). These results suggest that OCTN1 has differential role from OCTN2 or -3 by transporting other compounds such as organic cations as substrate. Transport of carnitine by rat OCTN2, which was also termed CT1, showed similar affinity (25 μM) (42) to that of mouse OCTN2 (22.1 μM). Furthermore, human OCTN2-mediated carnitine uptake was analyzed by means of the Eadie-Hofstee plot (22.1 μM) (42) to that of mouse OCTN2 (22.1 μM).

![Graphs and data](http://www.jbc.org/)

**FIG. 7. Concentration dependence of TEA uptake by OCTN1 and -2.** The uptakes of TEA by OCTN1 (a and c) or OCTN2 (b and d) were measured for 30 min at 37 °C in the Na⁺-containing transport buffer (pH 7.4). Open circles with dashed lines and open triangles with dotted lines represent the total and background uptakes obtained from cells transfected with the OCTNs and pcDNA3 plasmid vector alone, respectively. Solid lines represent the OCTNs-mediated uptake after subtraction of background uptake from the total uptake. OCTNs-mediated uptake was analyzed by means of the Eadie-Hofstee plot (c) of OCTN1 and OCTN2 (d). The results are shown as mean ± S.E. of three determinations.

### TABLE II

**Influence of several compounds on L-carnitine transport by OCTN2- and OCTN3-transfected HEK293 cells**

The uptake of L-[3H]carnitine was measured for 3 min at 37 °C in transport buffer (pH 7.4) containing each compound. The data were obtained by subtraction of the uptake by Mock-transfected cells from that by OCTN2- or OCTN3-transfected cells. Each value represents the mean ± S.E. of three determinations.

| Inhibitor          | Concentration | OCTN2 % of control | OCTN3 % of control |
|--------------------|---------------|--------------------|--------------------|
| Control            | 500 μM        | 100                | 100                |
| L-Carnitine        | 5             | 83.1 ± 1.14"       | 33.3 ± 0.84"       |
| Acetyl-L-carnitine | 5             | 67.3 ± 2.53"       | 20.7 ± 0.55"       |
| Butyryl-L-carnitine| 5             | 65.7 ± 1.23"       | 13.0 ± 0.24"       |
| Octanoyl-L-carnitine| 5           | 37.9 ± 0.69"       | 15.2 ± 0.34"       |
| Palmitoyl-L-carnitine| 5           | 28.4 ± 0.74"       | 53.8 ± 1.38"       |
| γ-Butyrobetaine    | 5             | 77.3 ± 0.96"       | 10.5 ± 1.26"       |
| γ-Butyrobetaine    | 50            | 43.8 ± 7.49"       | 5.6 ± 0.32"        |
| Glycinebetaine     | 50            | 86.8 ± 1.23"       | 103.3 ± 2.42"      |
| Glycinebetaine     | 500           | 55.5 ± 0.70"       | 83.5 ± 1.08"       |
| Choline            | 500           | 74.6 ± 4.59"       | 93.8 ± 3.12"       |
| Lysine             | 500           | 96.3 ± 1.44"       | 101.7 ± 4.14"      |
| γ-Aminobutyrate    | 500           | 95.0 ± 2.33"       | 94.5 ± 1.32"       |
| TEA                | 500           | 16.0 ± 1.31"       | 46.1 ± 1.15"       |

*a* Significantly different from the control uptake by Student’s $t$ test ($p < 0.05$).
carnitine in liver was significantly decreased in comparison with that of wild-type mice (24). Accordingly, the major Na\(^+\) -
dependent carnitine transporter in liver may be OCTN2, and the contribution of OCTN1 is minor. The reasons for this are
the very low activity of carnitine transport by OCTN1 (Fig. 3) and presumably lower expression in liver as observed in the
Western blot analysis (Fig. 2b). This is supported by the recent
finding of negligible transport of carnitine by rat OCTN1 (41).
Similarly, a significant contribution of OCTN2 to reabsorption of carnitine in kidney was demonstrated in human and jvs mice
(12–19, 24) despite strong expression of OCTN1 in mouse kid-
ney (Fig. 2, a and b). Very recently, it was reported that tar-
gested deletion of a region around OCTN1 gene as well as
OCTN2 gene caused many abnormalities related to lipid me-
tabolism in mice (45). Interestingly, the phenotypic abnormal-
ities were not improved by carnitine administration. The symp-
toms shown in OCTN2-defective jvs mice were improved by
carnitine administration (25). Taken all together, OCTN1
should play a distinct role from OCTN2 and -3 in the disposi-
tion of carnitine or related compounds.

Since mouse OCTN1 exhibited very low carnitine transport
activity, the substrate specificity of only mouse OCTN2 and -3
was examined by observing the inhibitory effect of various
compounds on carnitine transport (Table II). Strong inhibitory
effects were observed with carnitine-related compounds, in-
cluding acylcarnitines and \(\gamma\)-butyrobetaine, whereas lysine,
\(\gamma\)-aminobutyrate, or choline showed negligible or weak inhibi-
tory effect; these are consistent with observations in human
and rat OCTN2-mediated carnitine transport (9, 23, 42). De-
rivatives or precursor of carnitine showed stronger inhibitory
effects on carnitine transport mediated by OCTN3 than that
by OCTN2, whereas choline, TEA and glycinebetaine were
stronger inhibitors of OCTN2 than OCTN3. Accordingly, OCTN3 is
highly specific and has higher affinity for carnitine, and
OCTN2 has a rather broader specificity. However, since both
commonly accept carnitine and related compounds as sub-
strates and are present in kidney, they may have a common
role in the reabsorption of carnitine by mediating Na\(^+\) -
dependent and -independent transport, respectively. The other point
of interest is the pH dependence observed in carnitine trans-
port by OCTN2, but not by OCTN3 (Fig. 6). Earlier studies
suggested the existence of a specific carnitine transport system
in kidney, which showed Na\(^+\) and pH dependences (46, 47). The
luminal side of renal tubules is acidic, which may impair the
activity to some extent. At present, the physiological signifi-
cance of the apparent pH dependence observed in OCTN2-
mediated carnitine transport is not clear.

As discussed above, the contribution of OCTN3 to the reab-
sorption of carnitine in the kidney may not be great. Therefore
mouse OCTN3 was suggested to have a different role from
OCTN1 and OCTN2 on the basis of the absence of Na\(^+\) -
dependence, high specificity for carnitine, and limited tissue dis-
tribution (primarily in testis and a low level in kidney). Spermatozoa
are produced in the testis, mature by post-gonadal modifica-
tions (primarily in testis and a low level in kidney). Spermatozoa
were more inhibitory than carnitine itself on [\(^3\)H]carnitine trans-
port by mouse OCTN3 (Table II). The \(K_m\) value of acetyl-L-carnitine for mouse OCTN3 was 0.63 \(\pm\) 0.43 \(\mu\)M (data
not shown), which is lower than the value of carnitine (2.99
\(\mu\)M). Therefore, acylcarnitine may be a better substrate for
OCTN3. So far, no counterpart of mouse OCTN3 has been
found in human, and therefore, mice may have a distinct physi-
ology from human in carnitine disposition and/or carnitine-
related nutritional status. Alternatively, human OCTN1
and/or human OCTN2 may take the role of mouse OCTN3,
because both human OCTN1 and -2 were present in testis (9, 10).
Further studies on the role of the OCTN transporter in
testis and epididymis are needed.

In conclusion, OCTN1 and OCTN3 were cloned from mouse
as novel members of the OCTN transporter family. Compara-
tive studies of tissue distributions and functional characteris-
tics indicated that all the OCTNs play physiologically impor-
tant roles in carnitine transport, although OCTN1 showed very
low activity of carnitine transport. OCTN2 seems to be the
most physiologically important high affinity Na\(^+\) -
dependent carnitine transporter, operating for the reabsorption of
carnitine from urine as well as playing a major role in tissue dis-
tribution, whereas OCTN3 may be important in testis. OCTN1
and OCTN3 may contribute to reabsorption of carnitine in
kidney but were supposed to have other roles due to low activity
of OCTN1 and highly specific expression of OCTN3 in testis.
OCTN3 is a unique transporter with high specificity for carni-
tine, Na\(^+\) -independent transport activity, and predominant ex-
pression in testis. Furthermore, OCTN1 and -2 may be involved in
the distribution and elimination of organic cations in several
tissues. Accordingly, OCTN transporters seem to exhibit mul-
tifunctionality as carnitine and organic cation transport
systems.

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