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L-Carnitine transport in mouse renal and intestinal brush-border and basolateral membrane vesicles

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Abstract

We characterized the uptake of carnitine in brush-border membrane (BBM) and basolateral membrane (BLM) vesicles, isolated from mouse kidney and intestine. In kidney, carnitine uptake was Na^+-dependent, showed a definite overshoot and was saturable for both membranes, but for intestine, it was Na^+-dependent only in BLM. The uptake was temperature-dependent in BLM of both kidney and intestine. The BBM transporter in kidney had a high affinity for carnitine: apparent $K_m = 18.7$ μM; $V_{max} = 7.85$ pmol/mg protein/s. In kidney BLM, similar characteristics were obtained: apparent $K_m = 11.5$ μM and $V_{max} = 3.76$ pmol/mg protein/s. The carnitine uptake by both membranes was not affected within the physiological pH 6.5–8.5. Tetraethylammonium, verapamil, valproate and pyrilamine significantly inhibited the carnitine uptake by BBM but not by BLM. By Western blot analysis, the OCTN2 (a Na^+-dependent high-affinity carnitine transporter) was localized in the kidney BBM, and not in BLM. Strong OCTN2 expression was observed in kidney and skeletal muscle, with no expression in intestine in accordance with our functional study. We conclude that different polarized carnitine transporters exist in kidney BBM and BLM. L-Carnitine uptake by mouse renal BBM vesicles involves a carrier-mediated system that is Na^+-dependent and is inhibited significantly by specific drugs. The BBM transporter is likely to be OCTN2 as indicated by a strong reactivity with the anti-OCTN2 polyclonal antibody.

Keywords: Carnitine; Membrane transport; OCTN2; Mouse; Renal membrane vesicle; Intestinal membrane vesicle

1. Introduction

Carnitine, β-hydroxy γ-*N*-trimethylammonium butyrate, is an essential cofactor for the transfer of long-chain fatty acids from the cytosol into mitochondria for subsequent β-oxidation. Another well established function of carnitine is the maintenance of the cellular coenzyme-A pool [1]. In humans, L-carnitine is obtained from dietary sources, primarily meat and dairy products, and produced endogenously from the amino acids lysine and methionine, with both sources contributing to plasma and tissue levels.

The major site of L-carnitine absorption is the small intestine but the mechanism of intestinal uptake of L-carnitine is unclear. Earlier studies using intact intestinal tissue preparations suggested that intestinal L-carnitine uptake occurred by a carrier-

Abbreviations: BBM, brush-border membranes; BLM, basolateral membranes; OCTN2, novel organic cation/carnitine transporter 2; FSRFA, fast sampling rapid filtration apparatus; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; TEA, tetraethylammonium; SDS–PAGE, sodium dodecyl phosphate–polyacrylamide gel electrophoresis

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mediated process [2], but in a subsequent study using rat intestinal brush-border membrane (BBM) vesicles, it was concluded that the uptake process is a passive diffusion [3]. Thus, the mechanism of the L-carnitine uptake process remains controversial. More recently, McCloud et al. [4] showed that the L-carnitine uptake by intestinal epithelial cells (Caco-2) involves a carrier-mediated system (K_m of 45 μM) that is temperature-, Na^+- and energy-dependent.

Dietary intake and carnitine biosynthesis are responsible to maintain the carnitine body stores in mammals [5]. Carnitine is eliminated as free carnitine or acylcarnitine almost exclusively by the kidneys, where it is filtered and reabsorbed by $>95\%$ in the proximal tubule [1]. The kidney has in fact a crucial role in homeostatic regulation of carnitine concentration in body fluids. The carnitine reabsorption appears to be regulated such that normal plasma carnitine concentration (30–90 μM) is maintained without significant loss of carnitine in the urine [6]. When carnitine reabsorption is impaired, e.g. in patients with primary carnitine deficiency [7], the plasma and tissue carnitine concentrations decrease and clinical symptoms of carnitine deficiency can develop, underlining the importance of the renal carnitine transport system. Solute reabsorption by the kidney is a multistep process implying translocation of the substrate across the luminal and removal to the circulation by passage across the anti-luminal membrane (basolateral membrane, BLM) [8]. For carnitine, the renal transport system has been characterized in rat kidney BBM vesicles [8,9]. These studies revealed Na^+-dependent, saturable transport of L-carnitine, with K_m of 55 μM [8] and two transport systems with K_m values of 17 μM and 15 mM [9]. The transport could be inhibited by structural analogues such as D-carnitine, butyrobetaine, L-acetyl-carnitine but not by glycinebetaine. No study of carnitine transport in mouse apical or BLM vesicles has been reported.

Deficiency states occur when dietary sources are inadequate (such as in vegetarian diets), gastrointestinal absorption is decreased, renal losses are excessive, or endogenous production is inadequate secondary to an enzyme deficiency. Primary systemic carnitine deficiency (SCD; OMIM 212140) in human patients causes severe pathological symptoms such as cardiomyopathy, muscle weakness, fasting hypoglycemia and sudden death [10,11]. It is also characterized by excessive losses of intestinal and urinary carnitine [12]. SCD is transmitted in an autosomal recessive manner. It is caused by mutations in the OCTN2 gene which encodes a carnitine and organic cation transporter (OCT) cloned recently [13,14]. The OCTN2 protein has 557 amino acids with 12 putative transmembrane domains. Until now, three transporters of carnitine have been identified, and called OCTN1, OCTN2 and OCTN3. They belong to the OCT family and have different characteristics, namely affinity and capacity for carnitine transport, energization of transport, and sensitivity to inhibitors. These transporters are expressed in several tissues, but the precise intracellular localization is unknown.

A mouse model known as *jvs* or 'juvenile visceral steatosis' shows the same characteristics as the human SCD [15,16]. The homozygous *jvs* mutants show a microvesicular fatty infiltration of the viscera, and a significant cardiomyopathy. These mice have low levels of carnitine in the serum, the liver and the skeletal and cardiac muscles. Deficiency of carnitine transport has been demonstrated in the renal tubules, fibroblasts and the hepatocytes (absence of the high-affinity transporter, [17]). Recently, it has been confirmed that the gene for the mouse carnitine transporter OCTN2 (located on chromosome 11) is mutated in the *jvs* strain. In this mutation, leucine 352 is changed to arginine in the sixth transmembrane domain of OCTN2 [18].

A study of the physiology of L-carnitine transport in mice is important to understand the nature of human SCD and its mouse counterpart in the *jvs* model and to confirm the apical and/or BLM localization of OCTN2 in both kidney and small intestine. Our hypothesis is that different polarized carnitine transporters may exist in BBM and BLM. The rationale is based largely on analogy with other transport systems, such as renal α-ketoglutarate transporter [19] and lysine transporter implicated in lysinuric protein intolerance [20]. The properties of the two steps in carnitine absorption, transport across the renal and intestinal BBM and BLM, were evaluated by measuring the uptake of labeled substrate using purified membrane vesicles. Our results show that L-carnitine uptake by mouse renal

BBM vesicles involves a Na^+-dependent carrier that is likely to be OCTN2.

2. Materials and methods

2.1. Materials

L-[Methyl-^{3}H]carnitine hydrochloride (82 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). D-[1-^{3}H(N)]Glucose (30 Ci/mmol) was purchased from New England Nuclear (Guelph, Canada). The drugs tetraethylammonium (TEA), valproate, verapamil and pyrilamine and the other reagents were obtained from Sigma-Aldrich Chemicals (Oakville, Canada).

2.2. Membrane vesicle preparation

BBM vesicles were isolated from the renal cortex and the proximal half of the small intestine of mice (strain C57-BL/6J), by the Mg^{2+} precipitation method described by Hauser et al. [21]. Renal cortex and intestine were immediately removed and rinsed with ice-cold 0.9% NaCl solution. BBM from renal cortices and intestinal mucosal scrapings were prepared as previously described [22]. BLM vesicles from renal cortices and intestinal mucosal scrapings were prepared by a Percoll density gradient centrifugation method as described by Edwards et al. [19].

2.3. Biochemical assays

The protein determination was done by the method of Lowry et al. [23] using serum albumin as a standard. The degree of purity of membranes was assessed by measuring the enrichment of marker enzymes for the BBM, alkaline phosphatase [24], and for the BLM, Na^+,K^+-ATPase [25]. For the BBM, the relative enrichment of alkaline phosphatase over the original homogenate was 7.5 and 8 for intestine and kidney, respectively. For the BLM, the enrichment factor of Na^+,K^+-ATPase was 8 for intestine, and 8.5 for kidney. These values compare closely to those obtained in rabbits [26] and are generally considered as satisfactory.

2.4. Uptake studies

Uptake of [^{3}H]carnitine was done manually with manifold using cellulose nitrate filters, 0.65 μm pore size from Sartorius, Göttingen, Germany, or measured by a rapid filtration method using the FSRFA (fast sampling rapid filtration apparatus) as described by Malo and Berteloot [27]. BBM and BLM vesicles were usually resuspended in a final concentration of 15–20 mg of protein/ml in the final resuspension buffer (50 mM Tris–HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 0.1 mM $MgSO_4$, 250 mM KCl, 125 mM mannitol, pH 7.5), and 20 μl/0.5 ml incubation medium (final concentrations: 50 mM Tris–HEPES, 0.1 mM $MgSO_4$, 100 mM KCl, 150 mM NaCl, 125 mM, mannitol, pH 7.5) was used. The sodium-dependent uptake was determined by subtracting the sodium-independent (KCl only, 250 mM) uptake from the sodium-dependent (KCl 100 mM+NaCl 150 mM) uptake. (A) Manual uptake: L-[^{3}H]carnitine at 50 μM was added to the incubation medium and the reaction started by addition of membrane vesicles. After different incubation times, the uptake was stopped by adding 2 ml of ice-cold stop solution (cold resuspension buffer); the filters were washed three times with 2 ml ice-cold stop solution before radioactivity counting. (B) Uptake with FSRFA: this apparatus allows for sampling of up to 18 aliquots from the same incubation mixture and fully automates the sequence of vesicle injection and mixing with the incubation medium, samplings, washings, and filtrations. Carnitine uptake as a function of time was determined using nine aliquots of 50 μl each at different time intervals and processed as described in [27]. For both approaches, corrections were made for [^{3}H]carnitine bound to the filters in the absence of vesicles which was always $< 0.1\%$ of added counts; the functional integrity of BBM vesicles was verified by testing D-glucose uptake by renal and intestinal preparations ($v_i = 2.63 \pm 0.3$ pmol/mg protein/s for kidney, and $v_i = 2.15 \pm 0.24$ pmol/mg protein/s for intestine).

2.5. Assays with xenobiotic inhibitors

Membrane vesicles were prepared as described above. The incubation medium contained (final con-

centrations) 50 μM L-[^{3}H]carnitine (1 μCi per assay), 50 mM Tris–HEPES, 0.1 mM $MgSO_4$, 100 mM KCl, 150 mM NaCl, 125 mM mannitol, pH 7.5, and 500 μM of the xenobiotic. Transport was started by adding 6 μl of membrane suspension to 48 μl of preheated incubation medium (37°C). The reaction was stopped after 8 s for BBM, and 4 s for BLM.

2.6. Membrane localization and tissue distribution study by Western blot analysis

Rabbit polyclonal antibodies were raised against a synthesized polypeptide of mouse OCTN2 through Research Genetics, Inc. (Huntsville AL, USA). The amino acid sequence used was QWQIQSQTRMQKDGEESPT corresponding to amino acids 532–550 of OCTN2. Mouse tissues were isolated and homogenized in 4 ml buffer containing 10 mM Tris–HCl and 50 mM mannitol (pH 7.4) using a polytron homogenizer. The solution was then dispersed ultrasonically. After addition of sample buffer 2× (4% sodium dodecyl phosphate (SDS), 20% glycerol, 200 mM dithiothreitol, 120 mM Tris, pH 6.8, and 0.002% bromophenol blue) to the tissue homogenate or to the membrane vesicles, the solution was mixed, the proteins were denatured in a boiling water bath for 5 min and used for Western blot analysis. The sample was separated by 7.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and proteins were transferred to polyvinylidene difluoride membrane Immobilon (Millipore, Bedford, MA, USA) which was incubated in phosphate-buffered saline (PBS) buffer (150 mM NaCl, 2.5 mM KCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 0.02% sodium azide and 12% skim milk. The membrane was incubated with polyclonal anti-peptide antibody (in PBS buffer containing 0.02% sodium azide and 1% bovine serum albumin) overnight, rinsed with the above buffer without skim milk (but with 0.1% Tween 20), and incubated with secondary antibody (goat anti-rabbit IgG, horseradish peroxidase-linked whole antibody, Sigma, St. Louis, MO, USA). The membrane was washed as before and the proteins were detected by a chemiluminescence detection method using BM Chemiluminescence ELISA Substrate (POD) (Boehringer Mannheim, Laval, QC, Canada). The molecular weights were estimated by using prestained SDS–PAGE standards, broad range (Bio-Rad Laboratories, Hercules, CA, USA).

3. Results

3.1. Stability of BBM and BLM vesicles

Since there have been reports on the instability of BBM in rabbits and rats [28] and since uptake experiments usually last for a few hours, we have first verified the stability of both mice BBM and BLM vesicles by measuring the initial rates of 50 μM L-carnitine uptake after 10, 20, 40, 60 and 120 min (not shown) after thawing membrane vesicles from liquid nitrogen. In BBM, the initial rates of L-carnitine transport remained constant for 10 min, and then decreased abruptly to 60%. In BLM, the L-carnitine transport remained constant for 60 min, and decreased later on. All experiments reported in the following studies have been performed on both BBM and BLM within 10 min after thawing from liquid nitrogen, as during this period, both these preparations would remain stable.

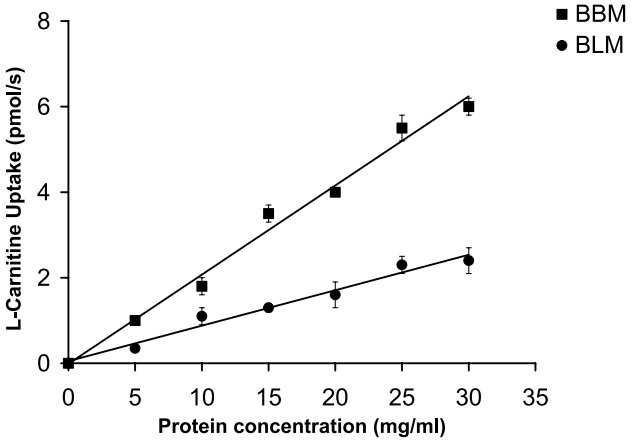

Fig. 1. Relationship between initial rates of L-carnitine uptake by renal BBM (squares) and renal BLM (circles) and membrane protein concentrations. Vesicles were prepared as described under Section 2. The initial rates of L-carnitine uptake have been determined at 37°C over the linear portion of the uptake time (8 s for BBM and 3 s for BLM, see Fig. 2). When not shown, the error bars were smaller than the symbols used. Linear regression analysis over the data points gave a correlation coefficient of 0.987 and 0.974 for BBM and BLM, respectively ($n = 2$ determinations).

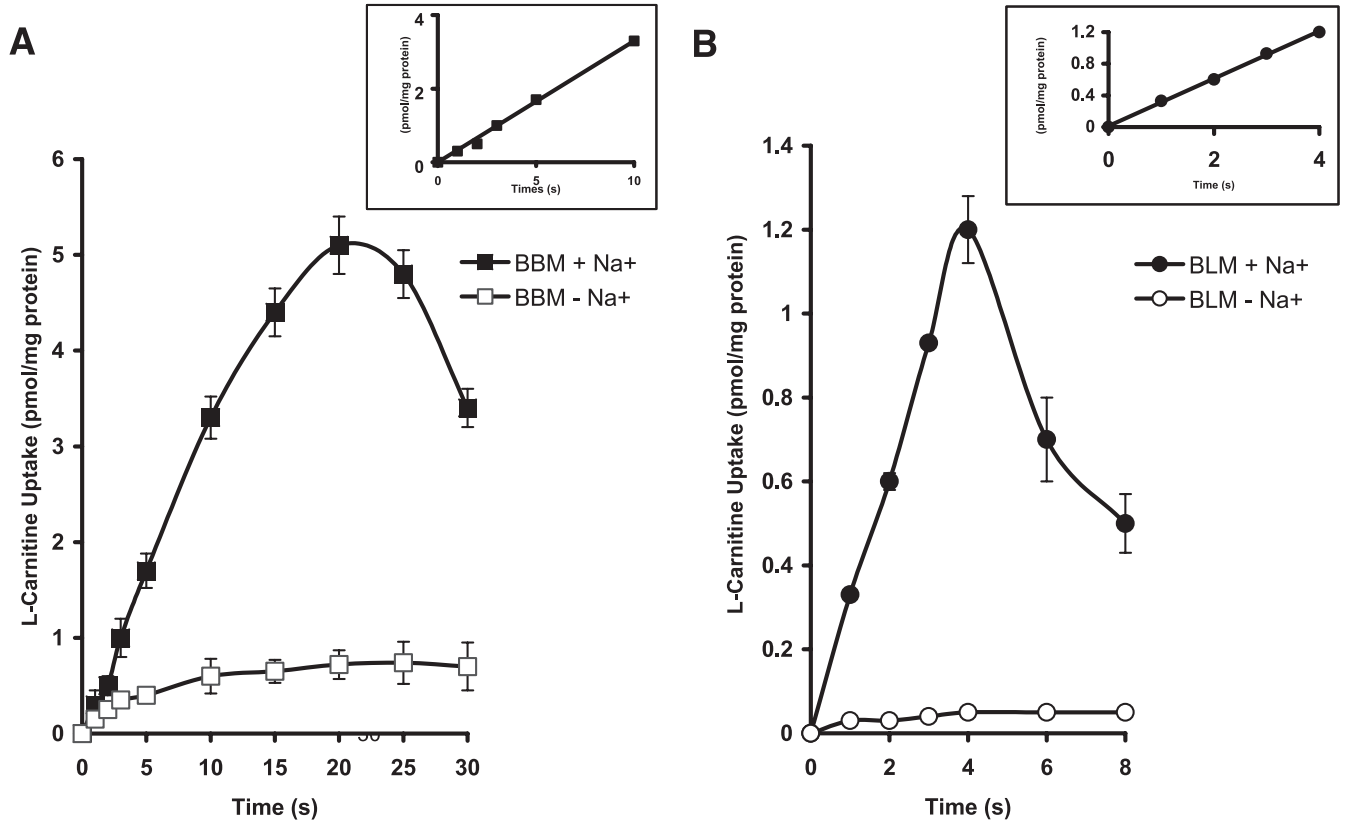

Fig. 2. Time course and Na^+ dependency of L-carnitine transport by mouse renal BBM (A) and BLM (B) at 37°C. L-[^{3}H]Carnitine uptake was determined using the FSRFA, as described under Section 2. The incubation medium contained (final concentrations) 50 μM L-[^{3}H]carnitine (1 μCi per assay), 125 mM mannitol, 50 mM HEPES adjusted with Tris to pH 7.5, 100 mM KCl and 150 mM NaCl (solid symbols) or 150 mM KCl (open symbols). Results are the mean ± S.D. from three different membrane preparations.

3.2. *Linearity of L-carnitine uptake with protein concentrations*

Renal BBM and BLM vesicles were resuspended at different protein concentrations ranging from 4 to 30 mg per ml. As demonstrated in Fig. 1, the initial rates of transport determined at a 50 μM L-carnitine concentration were linear over this range of protein concentrations for both membranes. All the following experiments have been performed using concentrations of 15–20 mg of BBM or BLM proteins per ml of resuspension medium.

3.3. *Time courses of L-carnitine uptake by renal BBM and BLM vesicles in mice using the FSRFA*

Fig. 2A,B depicts the initial rate of carnitine uptake by renal BBM and BLM vesicles. In BBM vesicles, an inwardly directed 150 mM Na^+ gradient caused a large stimulation of L-[^{3}H]carnitine uptake relative to that observed in the absence of Na^+ (Fig. 2A). The uptake was linear with time for 10 s (inset, $v_i = 0.33 \pm 0.05$ pmol/mg protein/s) and showed an overshoot at 20 s with a maximal accumulation of 5.2 pmol/mg protein. In BLM vesicles, the uptake of L-[^{3}H]carnitine was also much higher in the presence of a Na^+ gradient, but was linear only up to 4 s (inset, $v_i = 0.3 \pm 0.02$ pmol/mg protein/s) with a maximal accumulation of 1.2 pmol/mg protein, at the peak of the overshoot (Fig. 2B). These results indicated a Na^+-coupled carnitine transport in both membranes.

3.4. *Temperature dependency of L-carnitine uptake by renal BLM*

Carnitine uptake by renal BLM was very fast. In order to slow down the carnitine uptake, we deter-

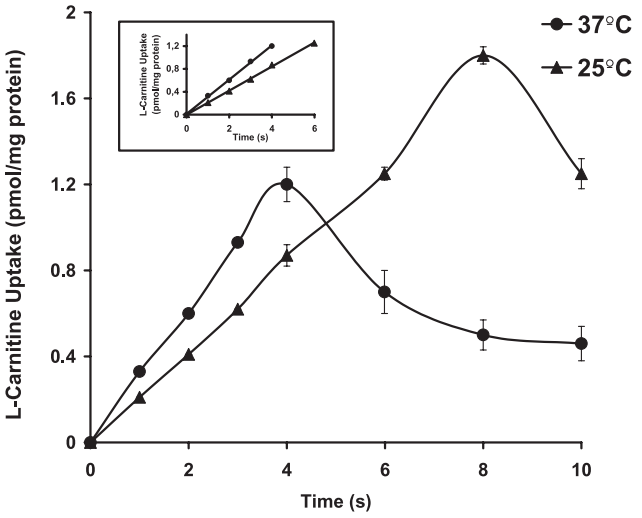

Fig. 3. Temperature dependency of L-carnitine transport by mouse renal BLM. Uptake was carried out at two temperatures: 37°C (circles) and 25°C (triangles). Conditions and procedures are similar to those described in Fig. 2, and under Section 2. Results are the mean ± S.D. from three different experiments.

mined the initial rate of carnitine uptake at two different temperatures, 37°C and 25°C. At 25°C, the uptake was linear with time for 6 s (inset, $v_i = 0.21 \pm 0.01$ pmol/mg protein/s), showed an overshoot at 8 s (Fig. 3) and was much higher in the presence of a Na^+ gradient (data not shown). Further experiments with renal BLM were performed at 25°C.

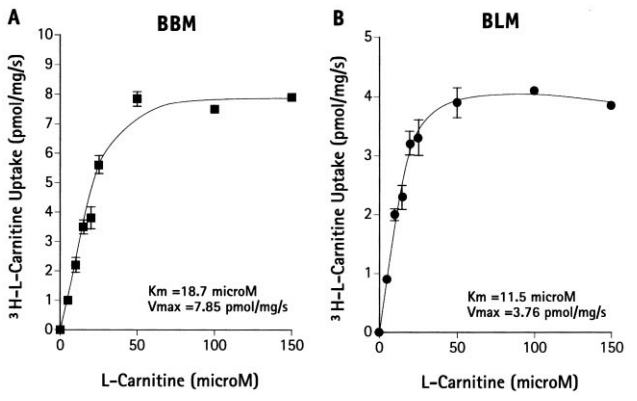

Fig. 4. Kinetics of Na^+-dependent L-carnitine transport by renal BBM (A) and BLM (B) vesicles. Initial rates of L-carnitine uptake into the membrane vesicles were measured after 8 s of incubation at 37°C in BBM, and after 5 s of incubation at 25°C in BLM, in the presence of a Na^+ gradient as described in Fig. 2. Carnitine concentrations are given on the abscissa. Results are the mean ± S.D. from two different experiments.

3.5. Kinetics of carnitine uptake by renal BBM and BLM vesicles

To determine kinetic parameters for Na^+-dependent carnitine uptake by renal BBM and BLM vesicles in mice, the initial rates of L-carnitine uptake were determined at different concentrations of unlabeled substrate (Fig. 4A,B). The kinetic parameters calculated by the Michaelis–Menten equation for one transport site were determined as described in [27], and were similar for the two membrane preparations.

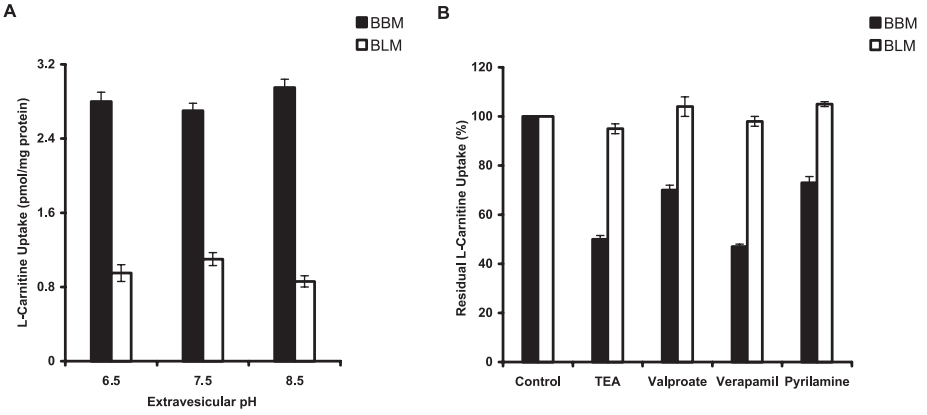

Fig. 5. Effect of extravesicular pH and xenobiotics on L-carnitine uptake by renal BBM (filled bars) and BLM (open bars). (A) The intravesicular pH was kept at pH 7.5 and the uptake medium pH was varied from 6.5 to 8.5. Initial rates of uptake were measured in the presence of a Na^+ gradient as described in Fig. 4. Results are the mean ± S.D. from three different experiments. (B) Effect of xenobiotics on L-carnitine transport by renal BBM and BLM. Membrane vesicles were incubated with 500 μM of each xenobiotic. Initial rates of carnitine uptake were measured in the presence of a Na^+ gradient as described in Fig. 4. Results are the mean ± S.D. from three different experiments.

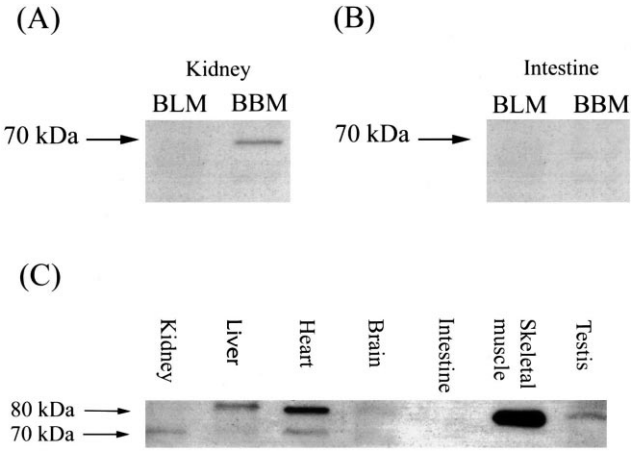

Fig. 6. Membrane localization in kidney (A), intestine (B) and tissue distribution (C) of OCTN2 by Western blot analysis. Anti-peptide antibody specific to OCTN2 was used. Renal or intestinal BBM and BLM from mouse (isolated as described under Section 2) (30 μg/lane) and tissue homogenates prepared from mouse kidney, liver, heart, lung, brain, skeletal muscle, testis and intestine (30 μg protein each) were separated on SDS–polyacrylamide gels (7.5%). To develop the Western blot, a peroxidase-coupled secondary antibody was used, and the reaction was visualized by chemiluminescence. Molecular weights (kDa) are shown on the left side.

The apparent affinity (K_m) of the BBM transporter for carnitine was 18.7 ± 2.4 μM with a maximum transport rate (V_{max}) of 7.85 ± 0.82 pmol/mg protein/s. Likewise, in BLM vesicles, the apparent K_m was 11.5 ± 1.8 μM and V_{max} was 3.76 ± 0.66 pmol/mg protein/s.

3.6. Effect of extravesicular pH

The effect of varying the extravesicular pH on renal BBM and BLM carnitine uptake was examined while keeping the intravesicular pH at 7.5. We found that changing extravesicular pH from 7.5 to either 6.5 or 8.5 did not significantly affect BBM and BLM carnitine uptake (Fig. 5A).

3.7. Effect of xenobiotics on L-carnitine transport by membrane vesicles

The ability of some drugs interacting with OCTN2 [29,30] to inhibit carnitine transport by mouse renal BBM and BLM was investigated (Fig. 5B). While TEA, valproate, verapamil and pyrilamine had no effect on the initial rate of carnitine uptake by renal BLM, these compounds inhibited significantly (inhibition between 27% and 54%) carnitine transport by renal BBM.

3.8. Renal membrane localization and tissue distribution of OCTN2

The mouse renal membrane localization of OCTN2 was investigated by Western blot (in view of the importance of kidney in the secretion and reabsorption of drugs, xenobiotics, and organic endogenous compounds, and because OCTN2 is better expressed in kidney). The BBM and BLM were isolated from mouse renal cortex and probed with the polyclonal antibodies. The mouse OCTN2-specific antibody labeled protein at the BBM with an apparent molecular mass of 70–80 kDa (Fig. 6A). BLM showed no immunoreactivity for the antibody (Fig. 6A).

The tissue distribution of OCTN2 was also determined by Western blot analyses (Fig. 6C). The polyclonal antibodies recognized proteins of 70 and/or 80 kDa in immunoblot of homogenate proteins from various tissues. Expression profile of OCTN2 protein

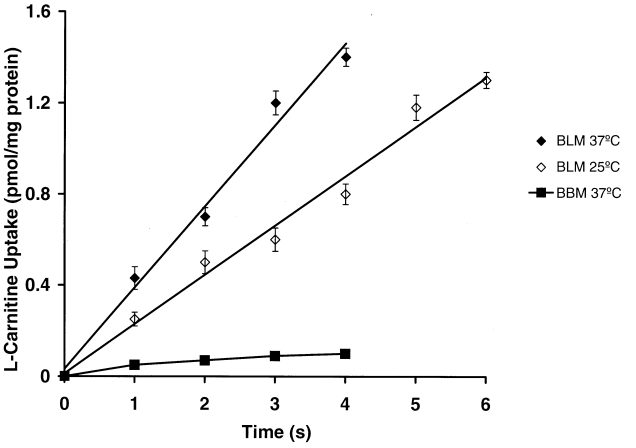

Fig. 7. Time course and temperature dependency of Na^+-dependent L-carnitine transport by mouse intestinal membrane vesicles. Carnitine uptake by BBM (squares) (v_i = 0.024 pmol/mg protein/s) and BLM (filled lozenges) (v_i = 0.36 ± 0.01 pmol/mg protein/s) at 37°C was measured in the presence of a Na^+ gradient. Carnitine uptake by mouse intestinal BLM was also measured at 25°C (open lozenges) (v_i = 0.22 ± 0.02 pmol/mg protein/s) in the presence of a Na^+ gradient. Conditions and procedures are similar to those described in Fig. 2, and under Section 2. Each point is the mean ± S.D. of three different experiments.

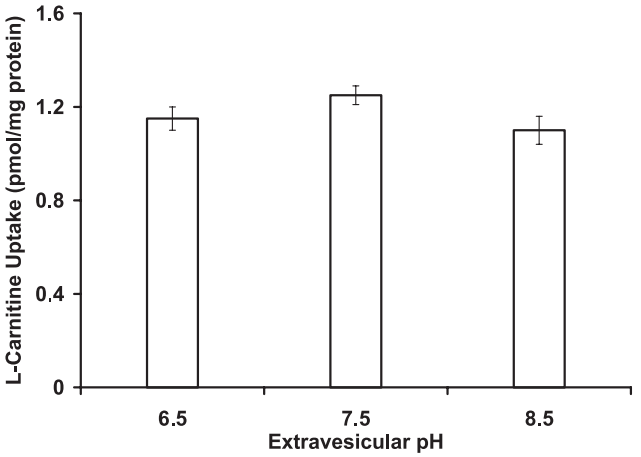

Fig. 8. Effect of extravesicular pH on L-carnitine uptake by intestinal BLM. Experimental conditions are similar to those described in Fig. 5A.

was broad (expression in kidney, liver, heart and skeletal muscle, but not in brain, lung, intestine and testis), and strong in skeletal muscle.

3.9. Carnitine uptake by intestinal BBM and BLM vesicles

Similar studies as described for kidney were done for intestine. The purity, stability and functional integrity of intestinal membrane vesicles were comparable to those found in kidney (data not shown). The time courses of L-carnitine uptake and the influence of Na^+ and temperature (37°C and 25°C) on carnitine transport by intestinal BBM and BLM were analyzed. In the presence of Na^+, carnitine uptake was different in the two membranes (Fig. 7). In intestinal BBM vesicles (filled squares), carnitine accumulation showed very low values (Fig. 7) while in BLM vesicles (filled lozenges), carnitine uptake was linear up to 4 s in the presence of a Na^+ gradient (Fig. 7). In the absence of Na^+, there is no carnitine uptake in both membranes (not shown). The temperature effect showed that at 25°C (Fig. 7, open lozenges), the uptake was linear with time for 6 s. Moreover, the carnitine uptake by intestinal BLM was pH-independent since there were no significant differences in uptake values when extravesicular pH was varied between 6.5 and 8.5 (Fig. 8). Elsewhere, intestinal membrane localization studied by Western blot showed an absence of immunoreactivity between OCTN2 antibodies and both BBM and BLM (Fig. 6B).

4. Discussion

We examined, for the first time in mice, the characteristics of carnitine uptake into BBM and BLM vesicles from renal cortices and intestinal mucosal scrapings. The use of isolated membrane vesicles avoided the interference of carnitine metabolism or carnitine uptake by other cellular compartments. We investigated, moreover, the localization at the luminal or basolateral plasma membrane of a recently cloned carnitine transporter, OCTN2, to understand its function in kidney and intestine.

4.1. Characterization of L-carnitine transport in the mouse kidney

In the mouse kidney, we found that there is a Na^+-dependent carnitine uptake across both BBM and BLM membranes with similar kinetic properties. These parameters were close to those found in rat BBM by Rebouche and Mack [8] and to the carnitine transport high-affinity system found in rat BBM by Stieger et al. [9]. In our studies the alteration of extravesicular pH between 6.5 and 8.5 had a minimal effect on carnitine uptake by both BBM and BLM vesicles in mouse kidney. The examination of the extravesicular effect of pH on BBM carnitine uptake is physiologically relevant because the in vivo pH of the proximal tubular fluid (equivalent to the extravesicular pH in the mostly right-side-out BBM vesicles) decreases along the proximal tubule, from 7.4 in the early proximal convoluted tubule to about 6.5 in the late proximal straight tubule [8].

Although the qualitative aspects of carnitine transport were similar in the two membrane preparations from kidney, there were some differences in the time courses of carnitine uptake. First, under Na^+ gradient conditions and at 37°C, the period of linearity was shorter (4 s) in BLM vesicles than in BBM vesicles (10 s) which may be explained by a faster dissipation of the Na^+ gradient. Second, the initial rate of carnitine uptake was more than two times higher in BBM than in BLM vesicles (Fig. 2A,B) which may reflect a higher concentration of carrier molecules within the apical membrane. The substrate specificity of the transporter (*s*) was also different in the two kidney vesicle preparations. The organic cations TEA (nicotinic, cholinergic antagonist), verapa-

mil (anti-angina, anti-arrhythmic), pyrilamine (antihistaminic), and the organic anion valproate (anticonvulsant, anti-epileptic) inhibited significantly carnitine uptake by BBM but not by BLM. This differential effect of various xenobiotics on carnitine uptake by renal membrane vesicles indicates clearly that there are different carnitine transporters in BBM and BLM from kidney. The lack of pH effect on carnitine transport by renal membrane vesicles suggests that the transport mechanism is not a cotransport of carnitine/H^+ in both mouse kidney BBM and BLM. This characteristic excludes an involvement of OCTN1 since this low-affinity transporter is pH-dependent in human [31] and rat [32] (although rOCTN1 does not mediate carnitine transport to any significant level) while pH dependency for mouse OCTN1 has not been previously tested [33]. The Na^+ dependency of carnitine transport in renal BBM and BLM vesicles excludes the implication of the very recently cloned mouse Na^+-independent OCTN3 [33].

Ohashi et al. [30] have tested the effect of various xenobiotics on kidney cells (HEK, human epithelial kidney) transfected with the carnitine transporter hOCTN2 and found a significant inhibition by TEA, valproate, verapamil and pyrilamine. These data suggested that carnitine transport in mouse renal BBM implicates OCTN2, a physiologically important Na^+-dependent high-affinity carnitine transporter in humans [13,14,30], rats [34,35] and mice [18,33], because OCTN2 has the unique feature of being the only carnitine Na^+-dependent high-affinity transporter within the OCT superfamily. OCTN2 transports carnitine in a Na^+-dependent manner, but transports organic cations such as TEA, a prototypical organic cation, in a Na^+-independent manner, conferring one unique characteristic among the Na^+-coupled organic solute transporters cloned so far [13,14]. In renal BLM, carnitine uptake was also a Na^+-dependent process but may involve a different carrier insensitive to any of the xenobiotics tested. In the presence of high levels of L-carnitine in the blood, the BLM transporter may have a regulatory role by preventing excessive accumulation of L-carnitine in the renal tubular cells [8]. Also, if this transporter is multispecific, it may be involved in the first step of elimination of drugs and xenobiotics in the urine.

Our hypothesis of polarized carnitine transporters in mouse kidney tubules was confirmed by Western blot analysis, which showed that OCTN2 is localized in BBM but not in BLM. Western blot indicated the proteins recognized by the antibody have molecular weights slightly over 70–80 kDa (Fig. 6) but close to the estimated size of OCTN2 deduced from the amino acid sequence (62 776 Da). This is probably referable to post-translational modification (such as phosphorylation or glycosylation). The strong expression of OCTN2 in kidney BBM supported its contribution to the reabsorption of carnitine from urine after glomerular filtration to maintain carnitine content in body. Very recently, Tamai et al. [36] showed that OCTN2 is localized on the apical membrane of renal tubular epithelial cells in both mice and rats [36]. Valproate, a therapeutic molecule for epilepsy, induced carnitine deficiency, and it was suggested that the deficiency is related to a transporter-mediated interaction between carnitine and the drug molecule [37,38]. Ohashi et al. [30] proposed that the competitive inhibition of hOCTN2-mediated carnitine transport by valproate may be one of the mechanisms of drug-induced carnitine deficiency. Since we found that valproate inhibited carnitine uptake by renal BBM and not by BLM, we suggest that this drug interacts with carnitine reabsorption by the OCTN2 transporter at the BBM level in kidney. The data do not exclude the possibility that the drug-induced carnitine deficiency during long-term treatment may be caused by inhibition of OCTN2-mediated carnitine transport in other tissues such as heart, skeletal muscle and liver, where OCTN2 is also strongly expressed. This correlates with the importance of carnitine function since more than 80% of carnitine is stored in muscle [39]. Heart and skeletal muscle are the two tissues which largely depend on carnitine to produce energy by long-chain fatty acid β-oxidation. More recently, Berardi et al. [40] have suggested the presence of the OCTN2 protein in the skeletal muscle by determining the properties of carnitine transport in plasma membrane vesicles from rat muscle. Only one previous study suggested that OCTN2 protein was present in the liver [17], based on similar kinetic parameters (K_m and V_{max}) found in cDNA OCTN2 transfected hepatocyte culture cells and membrane vesicles isolated from hepatocytes. Our tissue distribution is very close to that

obtained recently by Tamai et al. [33] on Western blot by using antibodies raised against the divergent COOH-terminal portions of the OCTN2 protein. So, two different specific anti-OCTN2 antibodies lead to the same results, although Tamai et al. [33] did not use skeletal muscle and intestine.

4.2. Characterization of L-carnitine transport in the mouse intestine

In the mouse intestine, we found that carnitine uptake by BBM was very low and Na^+-independent, while that by BLM was relatively high, temperature- and Na^+-dependent, but pH-independent (Fig. 7). This indicates that carnitine transport systems are also different between BBM and BLM in the mouse intestine. All these results obtained with membrane vesicles correlate with McCloud's study [4] using Caco-2 cells as an intestinal model. A Western blot conducted in these membranes showed no immunoreactivity between polyclonal antibodies against OCTN2 with intestine (homogenate fraction) (Fig. 6C) and BBM or BLM fractions (Fig. 6B), as in brain and lungs. So, Na^+-dependent carnitine transport in intestinal mouse BLM may be accomplished via a transporter other than OCTN2. This is supported by our finding that carnitine uptake by intestinal BLM is pH-independent (Fig. 8) while a known characteristic of mouse OCTN2 is to mediate carnitine transport in a pH-dependent manner [33]. In rat intestine, a carnitine transporter called CT1 (carnitine transporter type 1) was cloned earlier [35] but it corresponds to OCTN2 which is not expressed in mouse small intestine. Our results also suggest that there are different carnitine transporters in BBM and BLM from intestine based on the different carnitine uptake characteristics observed in these membranes (Fig. 7).

In summary, the present study shows the presence of different carrier-mediated systems for L-carnitine at the level of mouse renal and intestinal BBM and BLM. In kidney, carnitine uptake was Na^+-dependent, for both membrane preparations, but in intestine, it was Na^+-dependent only in BLM. The renal BBM carnitine transporter is likely to be OCTN2 as indicated by a strong reactivity with the anti-OCTN2 polyclonal antibody in Western blot, and by the significant inhibition of BBM carnitine uptake by the drugs TEA, verapamil, valproate and pyrilamine, in the uptake study. The tissue distribution of OCTN2 showed a broad expression profile of the protein with a strong expression in kidney, heart, skeletal muscle and liver, but with no expression in brain, lungs and small intestine which was in correlation with our functional study. Therefore, OCTN2 not only plays an important role in carnitine reabsorption in kidney, but may also be involved in carnitine distribution and organic cations elimination in several tissues.

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