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Functional Characterization of Intestinal L-Carnitine Transport

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Abstract. The carnitine transporter OCTN2 is responsible for the renal reabsorption of filtered L-carnitine. However, there is controversy regarding the intestinal L-carnitine transport mechanism(s). In this study, the characteristics of L-carnitine transport in both, isolated chicken enterocytes and brush-border membrane vesicles (BBMV) were studied. In situ hybridization was also performed in chicken small intestine. Chicken enterocytes maintain a steady-state L-carnitine gradient of 5 to 1 and 90% of the transported L-carnitine remains in a readily diffusive form. After 5 min, L-Carnitine uptake into BBMV overshot the equilibrium value by a factor of 2.5. Concentrative L-carnitine transport is Na^+-, membrane voltage- and pH-dependent, has a high affinity for L-carnitine (K_m 26 - 31 μM) and a 1:1 Na^+: L-carnitine stoichiometry. L-Carnitine uptake into either enterocytes or BBMV was inhibited by excess amount of cold L-carnitine > D-carnitine = acetyl-L-carnitine = γ-butyrobetaine > palmitoyl-L-carnitine > betaine > TEA, whereas alanine, histidine, GABA or choline were without significant effect. In situ hybridization studies revealed that only the cells lining the intestinal villus expressed OCTN2 mRNA. This is the first demonstration of the operation of a Na^+/ L-carnitine cotransport system in the apical membrane of enterocytes. This transporter has properties similar to those of OCTN2.

Key words: L-Carnitine — OCTN — Intestine — Enterocytes — Brush-border

Introduction

L-Carnitine transports acyl groups across the inner mitochondria membrane, where they become available for β-oxidation and also maintains the cellular pool of free coenzyme A. In man, endogenous L-carnitine synthesis occurs in liver, kidney and brain, but its precursor γ-butyrobetaine is synthesized in all tissues.

The importance of L-carnitine in metabolism becomes evident in patients with primary systemic L-carnitine deficiency (Kerner & Hoppel, 1998). Mutations in the gene encoding the renal Na^+/L-carnitine cotransporter caused the most severe form of that deficiency (Tamai et al., 1998; Nezu et al., 1999). L-Carnitine deficiency also occurs when either dietary sources (vegetarian diets) or endogenous production are inadequate, gastrointestinal absorption is decreased or under conditions where fatty acids become an important fuel, such as starvation and prolonged exercise. The role of dietary L-carnitine is unknown, but both, free and esterified L-carnitine are present in common foodstuffs and oral L-carnitine therapy produced marked clinical improvement in patients with primary systemic L-carnitine deficiency (Kerner & Hoppel, 1998).

In kidney, L-carnitine is reabsorbed by a Na^+- and energy-dependent mechanism (Rebouche & Mack, 1984; Stieger, O'Neill & Krähenbühl, 1995) that has been molecularly identified and named OCTN2 (Tang et al., 1999; Wu et al., 1999). Na^+-dependent L-carnitine transport has also been observed in several cell types (Shaw et al., 1983; Prasad et al., 1996; Nezu et al., 1999; Wu et al., 1999). However, there is controversy regarding the mechanisms that mediate intestinal L-carnitine absorption. Experiments carried out in in vivo preparations, intestinal rings, sacs and biopsies suggested that intestinal L-carnitine transport is carrier-mediated (Gudjonsson et al., 1985; Hamilton et al., 1986; Sekine et al., 1998). On the contrary, studies using either intestinal brush-border membrane vesicles (Li et al., 1990; Roque et al., 1996), isolated enterocytes (Gross, Henderson & Savaiano, 1986) or in vivo preparations (Gross & Henderson, 1984, Marciani

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et al.,1991), concluded that the uptake process was either a facilitated (Gross & Henderson, 1984; Gross et al., 1986) or a simple diffusion (Li et al., 1990; Marciani et al., 1991; Roque et al., 1996) mechanism. Gross & Savaiano (1993) suggested that L-carnitine is actively transported by enterocytes isolated from neonates but not by those from adult rodents. McCloud et al., (1996) reported that Caco-2 cells transport L-carnitine by a Na^+- and energy-dependent carrier-mediated system. Sekine et al., (1998) have cloned from rat small intestine a cDNA that encodes a protein (CT1) closely related to rat (Wu et al., 1999) and human (Tang et al., 1999; Wu et al., 1999) OCTN2. CT1 expressed in *Xenopus* oocytes mediates a high-affinity and Na^+-dependent L-carnitine transport.

In the current work we demonstrate the presence in the apical membrane of chicken enterocytes of an active Na^+-dependent L-carnitine transport system. Avian preparations are preferred to mammalian, as they survive longer in vitro.

Materials and Methods

Materials

L-(*methyl*-^{3}H)-Carnitine was purchased from Amersham. All the reagents used in the current study were obtained from Sigma Chemical, Madrid, Spain.

Cell Isolation and L-Carnitine Uptake Measurements

The experiments were performed in accordance with national/local ethical guidelines. Hubbard chickens, 4–6 weeks old, were killed by decapitation. Enterocytes were isolated by hyaluronidase incubation and incubated at 37°C, under the desired experimental condition (Calonge, Ilundain & Bolufer, 1989). Uptake was terminated by diluting 200 μl cell suspension in 800 μl ice-cold buffer and the cells were separated by centrifugation through a layer of an oil mixture, as described (Calonge et al., 1989). Cell pellets were extracted with perchloric acid (3%), mixed thoroughly and the suspension centrifuged. 100 μl aliquots of the supernatants were added to vials for scintillation counting. L-Carnitine uptake was calculated taking into account the trapped extracellular volume as previously estimated (Calonge et al., 1989) and the intracellular concentration of L-carnitine was evaluated by using a cell water volume of 3.6 μl/mg cell protein (Calonge et al., 1989).

Unless otherwise stated the incubation buffer contained, in mM: 100 NaCl, 1 $CaCl_2$, 60 mannitol, 3 K_2PO_4, 1 $MgCl_2$, 20 HEPES-Tris, pH 7.4 and 1 mg/ml bovine serum albumin. 10 mM fructose and 1mM L-glutamine were present in all the solutions as passively transported nutrients. The concentration of L-^{3}H-carnitine was 2.5 nM.

The pellet protein was measured by the method of Bradford (Bradford, 1976).

Brush Border Membrane Vesicle (BBMV) Preparation and L-Carnitine Uptake Measurements

BBMV were isolated from the small intestine of four to six week-old Hubbard chickens as described (Peral et al., 1995). Unless otherwise stated, the BBMV were loaded with a pH 7.5 buffer consisting of 140 mM mannitol, 50 mM KCl and 50 mM HEPES-Tris.

L-carnitine uptake was measured by a rapid filtration technique (Peral et al., 1995). Except where indicated otherwise, the uptake buffer consisted of 140 mM mannitol, 50 mM NaCl, 50 mM HEPES-Tris (pH 7.5) and 10 nM L-(^{3}H)-carnitine. The amount of protein in the assay tube ranged from 150 to 200 μg/100 μl of uptake buffer.

The protein was measured by the method of Bradford (Bradford, 1976).

Chromatographic Identification of Tissue Free L-Carnitine and Acid Soluble Acyl Carnitine

The enterocytes were incubated with 2.5 nM L-^{3}H-carnitine for 90 min and treated as described above, except that the cell pellets were extracted with 10% trichloroacetic acid. The acid was removed from the supernatant fraction by repeated extractions with 2.5 vols. of diethyl ether. Aliquots of the concentrated extract, which contained 2,000-10,000 cpm, were spotted on silica gel thin-layer chromatographic plates along with 5 μg nonradioactive L-carnitine and acetylcarnitine carriers. Ascending chromatography was performed on the plates using a solvent system consisting of (v:v), 55 methanol: 50 chloroform: 10 water: conc. 7.5 NH_4OH: cone. 2.5 formic acid. The lanes, to which radioactivity was applied, were cut into 0.5 cm sections. Each section was placed in a scintillation vial and counted. The remaining portion of the plate, which contained nonradioactive L-carnitine and acetylcarnitine standards, was exposed to iodine vapor. The relative mobilities were calculated from the visualized spots and from radioactivity measurements.

In Situ Hybridization

A 650-bp partial cDNA of rat OCTN2, generated by RT-PCR, was used to synthesize the OCTN2 riboprobes. Two micrograms of poly(A)$^+$ RNA isolated from rat small intestine were primed with random primer (to synthesize the first strand of cDNA) using a SuperScript preamplification system kit (Life Technologies) as described by the manufacturer. Two specific primers (CA F: 5′-GCTTGCGCTCACTGTACCAG-3′ and CA R: 5′-GACAG-GATGCTGCCAAGGCG-3′) based on the rat OCTN2 cDNA sequence (Sekine et al., 1998; Wu et al., 1999) were used. PCR conditions were: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. After initial denaturation, 35 cycles of PCR were run, with 10 min of final extension following the last cycle. The PCR products were gel-purified and subcloned using pGEM-T Easy Vector System (Promega). Plasmid DNA was analyzed by restriction digestion and sequenced. The sequence was identical to the CT1 and OCTN2 cDNA (Sekine et al., 1998; Wu et al., 1999).

To synthesize digoxigenin-UTP-labelled sense and antisense riboprobes, plasmid DNA containing the sequence of OCTN2 was linearized either by Pst I or by Apa I and transcribed in vitro with T7 or SP6 RNA polymerase (Amersham), respectively.

In situ hybridization on 50 μM-thick sections of the small intestine was performed as described (Nieto, Patel & Wilkinson, 1996). The sections were hydrated through methanol series, permeabilized with PBS containing 0.1% Tween20 (PBTW) and treated with proteinase K (5 μg/ml PBTW). Thereafter, the sections were refixed with 4% paraformaldehyde in PBTW and rinsed with PBTW. Afterwards, they were incubated with prehybridization solution (50% formamide; 2% blocking powder (Roche Molecular Biochemicals), 0.1% Triton X-100, 0.1% CHAPS (Sigma), 1 mg/ml tRNA, 5 mM EDTA, and 100 μg/ml heparin) for 16 hours and then DIG-labeled probes were applied at 37°C overnight. After the

sections were washed to low stringency at room temperature, they were washed three times for 30 min in 2 × SSC, 0.1% CHAPS; four times for 20 min in 0.2 × SSC, 0.1% CHAPS, blocked (3 hours, 4°C) with 20% fetal calf serum and with 0.7% blocking powder in KTBT (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 03% Triton X-100). The sections were incubated (overnight, 4°C) with alkaline phosphatase-conjugated anti-digoxigenin antibody. After removal of excess antibody, the alkaline phosphatase activity was detected by incubation in the dark with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer). The sections were photographed with an Olympus Provis Ax 70 (Japan) microscope under Nomarski optics.

Statistics

Data are presented as mean ± SEM. In the figures, vertical bars representing the SEM are absent when SEM is less than symbol height. Comparison between different experimental groups was evaluated by the two-tailed Student's t-test.

Results

Binding *vs.* Transport of L-(^{3}H)-carnitine in Intestinal BBMV

Nonspecific binding of the solute to the vesicle surface, which causes an overestimation of transport into the vesicle, was calculated as the uptake of L-carnitine by the vesicles at infinite osmolarity. At 180 min, increasing medium osmolality decreased L-carnitine uptake (Fig. 1), indicating that L-carnitine is taken up into an osmotically sensitive vesicular space. The relationship between uptake and the reciprocal of osmolality were fitted to a straight line. The intercept on the ordinate (zero intravesicular volume) is a measurement of nonspecific binding. The binding of L-carnitine measured in the presence and absence of a Na^+ gradient represents 47 and 30% respectively, of the L-carnitine uptake measured in standard conditions.

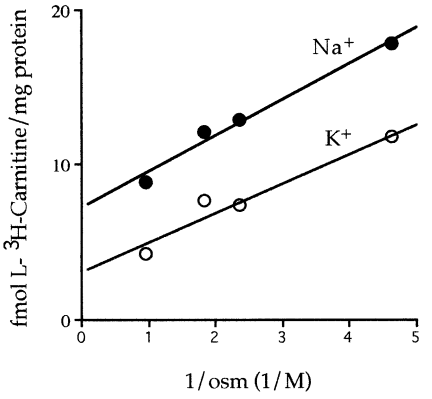

Fig. 1. External osmolality and L-(^{3}H)-carnitine uptake into BBMV. Medium osmolality was increased by the addition of mannitol. Uptake of L-(^{3}H)-carnitine was measured during 180 min with or without extravesicular Na^+ (Na^+ isosmotically replaced by K^+). The composition of the buffers is given in Methods. Lines were calculated by linear regression analysis. Mean values ± SEM, $n = 4$.

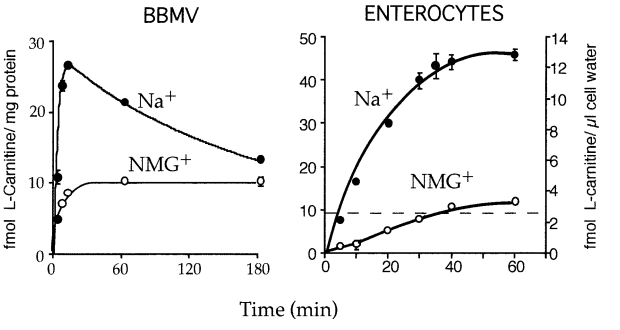

Fig. 2. Time course of L-carnitine uptake into either isolated enterocytes or BBMV. L-^{3}H-carnitine uptake was measured in the presence or absence of external Na^+ (Na^+ replaced isosmotically by NMG^+). The dashed line represents the uptake value expected at equilibrium. Mean ± SEM of 5 independent experiments.

Time Course and Ion-Dependence of L-Carnitine Uptake into Either Isolated Enterocytes or BBMV

L-(^{3}H)-Carnitine is taken up by the enterocytes from nominally Na^+-free medium and at steady state (60-min incubation), L-carnitine cell concentration approaches that in the incubation buffer (Fig. 2). Extracellular Na^+ stimulates L-carnitine transport and the uptake follows an exponential time course, reaching a steady-state accumulation value of 46 ± 3 fmol/mg protein (12.8 ± 0.4 nM), which corresponds to an inside: out ratio of 5:1. These observations suggest the presence of an L-carnitine active transport system.

Measurements in BBMV revealed that an inwardly-directed Na^+ gradient stimulated the amount of L-(^{3}H)-carnitine associated with the vesicles, overshooting the equilibrium value by a factor of about 2.4 at 5 min. No overshoot was observed in nominally Na^+-free conditions (Fig. 2). From the value of L-carnitine reached at the equilibrium in Na^+-free conditions, the estimated average volume of the vesicles was 1.11 ± 0.01 µl/mg protein. The different binding obtained with in presence or absence of Na^+ (*see* Fig. 1) may explain the differences in steady-state uptake values (at 180 min).

Energy-Dependence of the L-Carnitine Uptake into Chicken Enterocytes

Cells were preincubated with 10 mM of either dinitrophenol or iodoacetic acid for 20 min at 37°C and uptake of L-^{3}H-Carnitine was measured thereafter for 10 min in presence of Na^+. Dinitrophenol and iodoacetic acid reduced L-carnitine uptake from 16.3 ± 2 to 7.6 ± 0.7 and to 6.4 ± 0.6 fmol/mg protein/10 min,

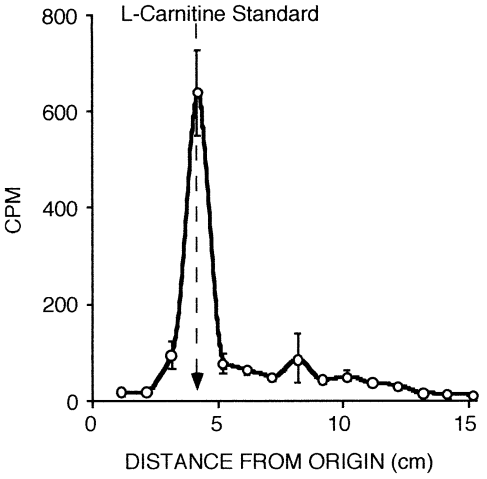

Fig. 3. Thin layer chromatographic analysis of trichloroacetic extracts from chicken enterocytes incubated with 2.5 nM L-^{3}H-carnitine for 90 min. Chromatographic procedures are described in Methods.

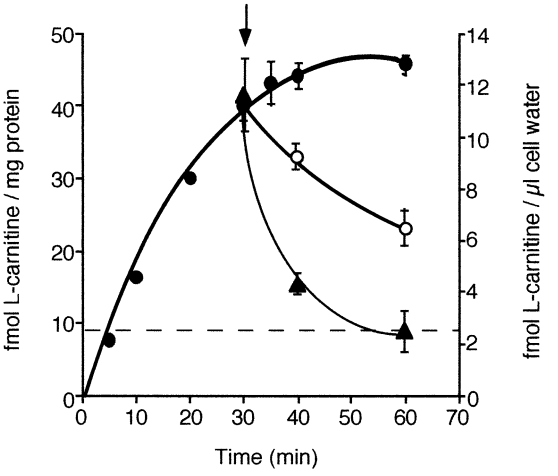

Fig. 4. Effect of either D-carnitine or temperature on steady-state cell L-carnitine concentration. Cells were allowed to accumulate L-^{3}H-carnitine(●). At the time indicated by the arrow, either 5 mM D-carnitine was added (▼) or the cells were transferred to an ice-bath (○). Other details as in Fig. 2.

respectively. At 0°C, L-carnitine uptake was undetectable.

Chromatographic Identification of Enterocyte Radioactivity

Once inside the cells, radiolabelled substrates can be either metabolized or bound to intracellular components. The nature of the cell radioactivity was determined by trichloroacetic acid-ether extraction, which separates free and short-chain acylcarnitines from long-chain acylcarnitines. Thin layer chromatography of the acid-soluble phase revealed a peak (Rf = 0.28), accounting for the 90% of the total applied radioactivity that migrated with authentic free L-carnitine (Fig. 3). These observations indicate that the L-carnitine accumulated by chicken enterocytes remains in the free form for at least 90 min and provide additional evidence that chicken enterocytes transport L-carnitine against a concentration gradient of free L-carnitine.

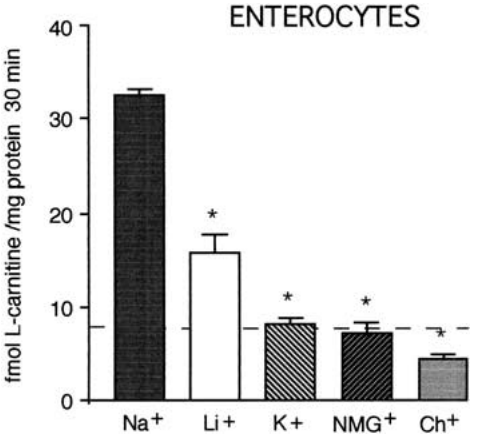

Fig. 5. Effect of cations on L-^{3}H-carnitine uptake into enterocytes. Uptake was measured with and without extracellular Na^+ (Na^+ replaced isosmotically by the indicated cations). NMG^+: N-methylglucamine, Ch^+: choline. Other details as in Figure 2.

Effect of D-Carnitine and Temperature on Enterocyte Steady-state L-^{3}H-Carnitine Uptake

To further corroborate that we were dealing with free L-carnitine transport and that the observed 5:1 ratio was not an artefact, the active cell accumulation of L-^{3}H-carnitine was interrupted, after a period of 30 min, by either adding 5 mM D-carnitine or transferring the cells into an ice bath, and the release of L-^{3}H-carnitine was monitored. Fig. 4 shows that under these two experimental conditions, the cells released the accumulated L-carnitine, and after 30 min the cell L-carnitine concentration approached that of the extracellular medium.

Effect of Na^+ Chemical Gradient and Electrical Membrane Potential on L-(^{3}H)-Carnitine Uptake into Either Enterocytes or BBMV

The iso-osmotic replacement of Na^+ with either K^+, N-methylglucamine$^+$ or choline$^+$ abolished cell L-carnitine accumulation (Fig. 5). There was substantial active transport of L-carnitine with Li^+ replacing Na^+, suggesting that Li^+ substitute for Na^+ to a significant extent. Ouabain (1 mM), a Na^+, K^+, AT-Pase inhibitor, abolished concentrative L-carnitine uptake into chicken enterocytes (*data not shown*). High external potassium plus 20 µM valinomycin, an experimental condition that reduces electrical mem-

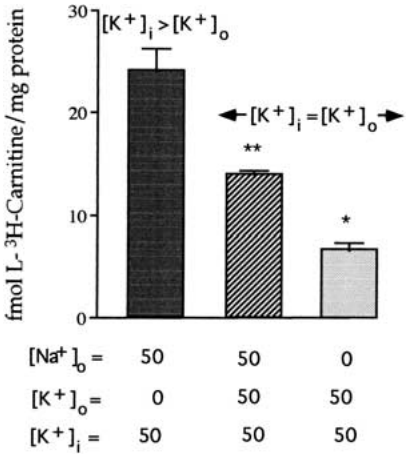

Fig. 6. Effect of electrical membrane potential and/or Na^+ chemical gradient on L-(^{3}H)-carnitine uptake into BBMV. The BBMV were loaded with a buffer consisting of, in mM, 140 mannitol, 50 KCl and 50 HEPES-Tris (pH7.5). The uptake buffer contained (in mM): 50 HEPES-Tris (pH7.5), 0.045 valinomycin and the indicated concentrations of Na^+ and K^+. Osmolality was maintained with mannitol. ■, electrochemical Na^+ gradient; ▨, chemical Na^+ gradient; ▒, absence of electrochemical Na^+ gradient. L-(^{3}H)-carnitine uptake was measured during 5 min. K_i and K_o *above* the *bars* indicates the relative $[K^+]$ (i.e., membrane potential). Mean values ±SEM, $n = 4$ *, $p < 0.001$; **, $p < 0.05$; as compared with electrochemical Na^+ gradient conditions.

brane potential, significantly ($p < 0.001$) decreased L-carnitine uptake into enterocytes from 42 ± 1.5 to 32 ± 1.6 fmol/mg protein ·30 min.

L-(^{3}H)-Carnitine uptake into BBMV was measured with or without an inwardly-directed electrochemical Na^+ gradient. Electrical membrane potential was created by an outwardly-directed K^+ gradient in the presence of valinomycin. When required, voltage across the membrane was brought to zero by equal internal and external K^+ concentrations in the presence of valinomycin. An inside-directed Na^+ gradient was created by adding 50 mM Na^+ to the extravesicular buffer, being the intravesicular buffer nominally Na^+ free. Na^+ chemical gradient was abolished by isosmotical replacement of Na^+ by mannitol in the extravesicular solution. Fig. 6 shows that membrane voltage-clamped conditions inhibited L-carnitine uptake and that this inhibition was further increased in nominally Na^+-free conditions.

All together, these observations suggest that both, the electrical membrane potential and the chemical Na^+ gradient energize L-carnitine uptake.

Effect of pH on L-(^{3}H)-Carnitine Uptake

In both, enterocytes and BBMV, external alkalization stimulated L-carnitine uptake, whereas it was inhibited by external acidification (Table 1). In the absence of a transmembrane pH gradient ($pH_i = pH_o = 8$), external alkalization also stimulated L-carnitine uptake.

Table 1. Effect of pH on L-carnitine transport in chicken enterocytes and BBMV

Enterocytes		BBMV		
pH_o	fmol/mg protein	pH_o	pH_i	fmol/mg protein
5.5	13* ± 0.3	5.5	7.5	6.0* ± 0.01
7.4	18 ± 2	7.5	7.5	20 ± 0.6
8.0	26* ± 2	8	7.5	33* ± 0.6
		8	8	34* ± 0.6

L-(^{3}H)-carnitine uptake into either chicken enterocytes (2.5 nM, 30 min) or BBMV (10 nM, 10 min) was measured in the presence of Na^+. Means ± SEM, of 5 independent experiments. *: $p < 0.001$ compared with values obtained at pH.

Effect of Unlabeled L-Carnitine, L-Carnitine Analogs and Organic Cations on L-Carnitine Uptake into Chicken Enterocytes and BBMV

Substrate specificity of the L-carnitine transport system was evaluated in terms of the inhibitory effect on the L-^{3}H-carnitine uptake. The measurements were carried out with or without external Na^+. Acetylcarnitine, palmitoyl-L-carnitine, betaine and γ-butyrobetaine compete with Na^+-dependent L-carnitine transport in several cell types (Shaw & Li, 1983; Gross & Henderson, 1984; Gross et al., 1986; Sekine et al., 1998, Shennan et al., 1998; Ohashi et al., 1999). Tetraethylammonium (TEA), alanine, histidine and choline$^+$ were chosen to test whether L-carnitine is transported by either the cationic or amino-acid transport systems. Table 2 shows that L-and D-carnitine, γ-butyrobetaine, acetylcarnitine, palmitoylcarnitine, betaine and TEA inhibited L-carnitine uptake, whereas GABA, alanine, histidine and choline$^+$ had no significant effect. The lipophilic organic cation verapamil, an inhibitor of OCTN2 (Ohashi et al., 1999) also significantly inhibited L-carnitine uptake.

Kinetic Study of the L-Carnitine Uptake into Enterocytes and BBMV

The concentration dependence of L-carnitine uptake into either isolated enterocytes or BBMV was measured in the presence of Na^+ at increasing concentrations of unlabeled L-carnitine (Fig 7). The shape of the curve suggested the existence of a saturable process and the data were analyzed by a nonlinear regression data analysis program (ENZFITTER). The values fit best ($r = 0.997$) a transport model describing a single saturable transport system plus a nonsaturable diffusion component:

$$v = (V_{max}S/K_m + S) + DS$$

Table 2. Specificity of L-carnitine transport in chicken enterocytes and BBMV

Modifier	Relative transport rates (% of control)	
	Cells	BBMV
None	100 ± 3	100 ± 1
250 μM		
L-Carnitine	18 ± 2[a]	13 ± 2[a]
D-Carnitine	18 ± 2[a]	14 ± 2[a]
Palmitoyl-L-Carnitine	28 ± 1[a]	34 ± 2[a]
L-Acetylcarnitine	34 ± 3[a]	18 ± 2[a]
γ-Butyrobetaine	13 ± 3[a]	15 ± 2[a]
Betaine	74 ± 6[a]	55 ± 4[b]
TEA	75 ± 5[a]	68 ± 6[c]
Alanine	100 ± 1	
Histidine	100 ± 1	
Choline	100 ± 1	
γ-Aminobutiric acid	100 ± 1	81 ± 3[c]
Verapamil	15 ± 5[a]	13 ± 4[a]
10 μM		
L-Carnitine	68 ± 4[a]	51 ± 5[b]
D-Carnitine	74 ± 5[a]	70 ± 5[b]
L-Acetylcarnitine	76 ± 1[a]	55 ± 5[b]
Palmitoyl-L-Carnitine	79 ± 1[a]	87 ± 3[d]
γ-Butyrobetaine	68 ± 6[a]	54 ± 4[b]
Betaine	92 ± 6	77 ± 3[c]

L-Carnitine uptake measured in the absence of modifiers was set at 100%. Other details as in Table 1. Means ± SEM of 5 independent experiments. [a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.02$, [d]$p < 0.05$, as compared with control (first row).

Table 3. Kinetic parameters of the Na^+/L-carnitine transport system measured in chicken enterocytes and in the BBMV isolated from the chicken small intestine.

	V_{max}	K_m	Hill coeff.	$(Na^+)_{0.5}$
Enterocytes	11 ± 0.1	26 ± 1	0.94 ± 0.01	22 ± 1
BBMV	13 ± 0.1	31 ± 1	1.04 ± 0.10	28 ± 1

The apparent K_m (in μM), $(Na^+)_{0.5}$ (in mM), V_{max} (pmol/μl cell water/1min) and the Hill coefficient (coeff.) values were calculated using nonlinear regression analysis. Means ± SEM of 5 independent experiments.

where v is initial rate of uptake; S, the external L-carnitine concentration; V_{max}, the maximal initial uptake rate; K_m, the Michaelis-Menten constant, and D, the apparent diffusion constant. The calculated apparent K_m and V_{max} values for L-carnitine transport are given in Table 3.

In BBMV L-carnitine uptake was also measured in the absence of Na^+ and the results showed a linear relationship with its extravesicular concentration. The difference between total L-carnitine uptake and that observed in the absence of Na^+ follows first-order kinetics.

The Eadie-Hofstee plot of the saturable component (Fig. 7, inserts) yielded in both cases a linear relationship, consistent with the existence of a single saturable L-carnitine transporter.

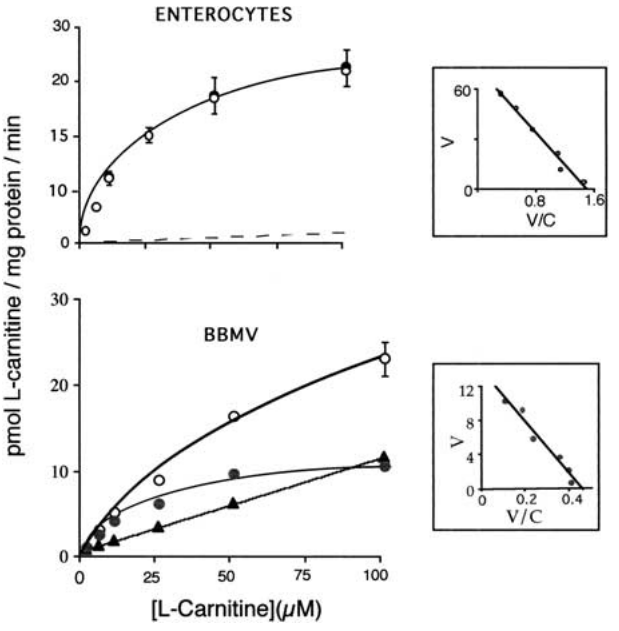

Fig. 7. One-minute L-carnitine uptake *vs.* increasing concentrations of external L-carnitine ranging from 0.1 to 100 μM. The values for the nonsaturable component (- - -) were determined by either fitting the total transport data (○) to a transport model describing a single saturable system (●) plus a nonsaturable component (▲, enterocytes) or by measuring L-carnitine uptake in Na^+-free conditions. *Inserts*: Eadie-Hofstee plot of the saturable component (total uptake minus diffusion component). 5 independent experiments.

Na^+: L-Carnitine Stoichiometry

Because the Na^+-dependent L-(^{3}H)-carnitine uptake into both, isolated enterocytes and BBMV, was stimulated by an inside-negative membrane potential, suggesting the transfer of positive charge across the membrane during the uptake process, the Na^+: L-carnitine stoichiometry was evaluated. We measured the dependence of the initial rate of L-(^{3}H)-carnitine uptake on the extravesicular concentration of Na^+. The Na^+-dependent component for the L-carnitine uptake was calculated by subtracting the uptake measured in the absence of Na^+ from that measured in its presence (Fig. 8). The Hill plot of the relationship between Na^+-dependent L-carnitine uptake and external Na^+ concentration (Fig. 8, inserts) is linear and gives an interaction coefficient close to 1 (*see* Table 3), which implies a 1:1 Na^+: L-carnitine stoichiometry. The apparent dissociation constant for the interaction of Na^+ with the transport system are given in Table 3.

In Situ Hybridization

The photomicrographs of chicken small intestine hybridized with an antisense riboprobe for rat OCTN2 (Fig. 9) revealed that OCTN2 mRNA ex-

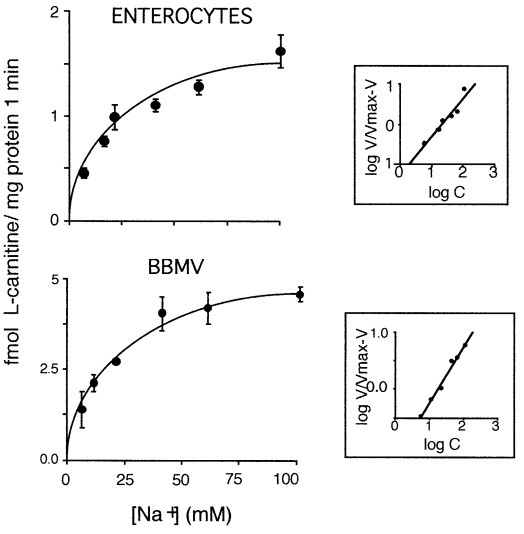

Fig. 8. Stoichiometry of Na^+:L-carnitine transport. 1 min L-^{3}H-carnitine uptake was measured as a function of 0–100 mM Na^+, with N-methylglucamine$^+$ replacing Na^+ isosmotically. Values are the Na^+ -dependent L-carnitine uptake. Mean values ± SEM, $n = 5$. *Insert*: Hill (log-log) plot. V_{max} was evaluated from the Eadie-Hofstee plot. Least-squares fit of the data yields a slope of 0.94 (stoichiometry) and x-intercept ($K_{0.5}$) of 22 mM, $r = 0.96$.

pression is restricted to the cells lining the villus, but it was absent from the crypt cells. The specificity of the hybridization signal was demonstrated by the low background labeling observed when the sense riboprobe was used on adjacent sections of the tissue.

Discussion

L-carnitine is not considered an essential nutrient for adult mammals because it can be synthesized. However, a significant amount of L-carnitine is provided by the diet and oral L-carnitine is successfully used to treat L-carnitine deficiency (Kerner & Hoppel, 1998). Whereas the renal L-carnitine transporter (OCTN2) has been well characterized and molecularly identified (Rebouche & Mack, 1984; Stieger et al., 1995; Tamai et al., 1998; Wu et al., 1999), the intestinal L-carnitine transport studies have provided conflicting results (Shaw & Li, 1983; Gross & Henderson, 1984; Gudjonsson et al., 1985; Gross et al., 1986; Marciani et al., 1991; Gross & Savaiano, 1993; McCloud et al., 1996, Roque et al., 1996) and previous studies were unable to show an active and Na^+-dependent L-carnitine transport activity in intestinal BBMV (Li et al., 1990; Roque et al., 1996). We present evidence demonstrating that chicken enterocytes have at its apical membrane a Na^+/L-carnitine cotransport system and that 90% of the transported L-carnitine remains in its free form for at least 90 min.

Evidence indicating that the energy derived from the membrane electrochemical Na^+ gradient drives transport of L-carnitine across the brush border against a concentration gradient is presented. Thus, both, enterocytes and BBMV, accumulated L-carnitine in the presence of an inwardly directed electrochemical Na^+ gradient. The Na^+-dependent L-carnitine uptake is electrogenic, because it was reduced by voltage-clamped conditions. Concentrative L-carnitine uptake into the cells was abolished by metabolic inhibitors (dinitrophenol and iodoacetic acid), ouabain or by Na^+ replacement by either choline$^+$, K^+ or NMG^+.

The Na^+/L-carnitine transporter responsible for the active transport of L-carnitine in the enterocytes is the same as that present in the isolated BBMV. Thus, in both preparations the transport system has the same substrate specificity, the same affinity for L-carnitine and for Na^+, the same L-carnitine V_{max} and a 1:1 Na^+:L-carnitine stoichiometry (*see* Table 3).

A 1:1 Na^+:L-carnitine stoichiometry has been measured in hOCTN2-transfected HEK293 cells (Ohashi et al., 1999), in human placental choriocarcinoma cells (Li et al., 1990) and in human kidney (Hamilton et al., 1986).

The K_m values obtained in the current work are similar to those reported for the cloned L-carnitine transporter, CT1, (Sekine et al., 1998); for Na^+/L-carnitine transport in kidney (Stieger et al., 1995; Huang et al., 1999), and in choriocarcinoma cells (Prasad et al., 1996); lower than that reported for rat kidney cortex (Rebouche & Mack, 1984; Yokogawa et al., 1999), NB-2a cells (Nalecz et al., 1995), lactating rat mammary tissue (Shennan et al., 1998) and Caco-2 cells (McCloud et al., 1996); an order of magnitude lower than that measured in human intestinal biopsies (Hamilton et al., 1986) and rat small intestine (Shaw et al., 1983; Gudjonsson et al., 1985), and higher than the values given for the cloned OCTN2 (Tamai et al., 1998; Ohashi et al., 1999) and for hepatoma HLF cells (Yokogawa et al., 1999).

As in human intestine (Shaw et al., 1983), Caco-2 cells (McCloud et al., 1996) and OCTN2-transfected HEK cells (Tamai et al., 1998, Ohashi et al., 1999), L-carnitine uptake into either enterocytes or BBMV shows no stereospecificity (*see* Table 2). In human hepatoma HLF cells (Yokogawa et al., 1999), placental choriocarcinoma cells (Prasard et al., 1996) and

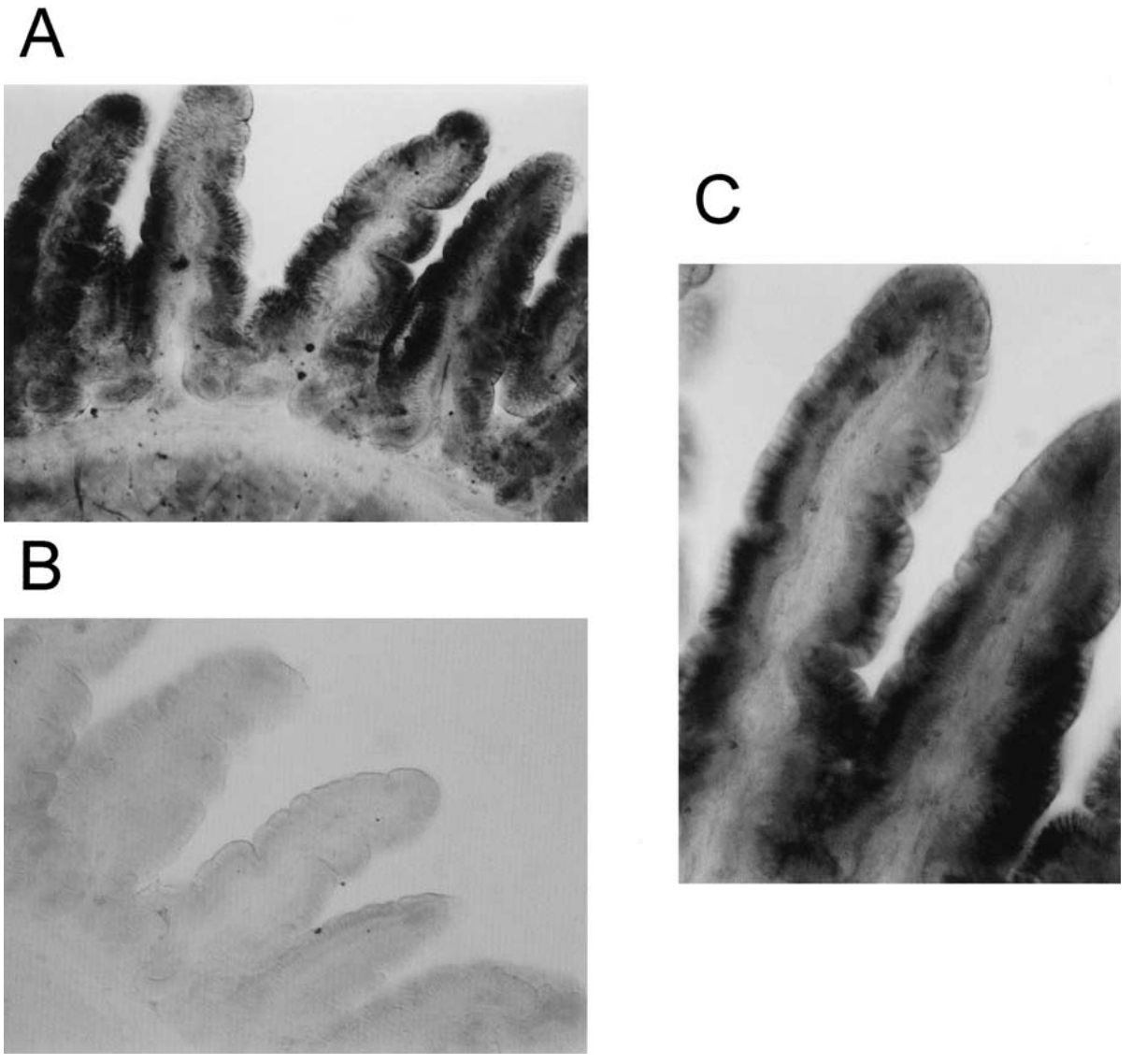

Fig. 9. Expression of OCTN2 mRNA in chicken small intestine. Panels *A* (×10), *B* (×10) and *C* (×40) are bright-field photomicrographs of chicken small intestine which have been hybridized in situ with either antisense (*A* and *C*) or sense (*B*) digoxigenin-UTP-labeled riboprobe for rat OCTN2 (*see* Methods section).

in rat kidney slices (Huth & Shug, 1980; Stieger et al., 1995), L-carnitine transporter favours the L-isomer. γ-butyrobetaine, acetylcarnitine, palmitoylcarnitine, TEA, betaine, and verapamil also inhibited L-carnitine uptake. It has been shown that OCTN2 transports TEA in a Na^+-independent manner (Ohashi et al., 2001) and it is inhibited by verapamil (Ohashi et al., 1999).

The pH-dependency of the L-carnitine transport process is similar to that of HLF cells (Yokogawa et al., 1999), that of the rat renal Na^+/L-carnitine cotransporter expressed in *Xenopus* oocytes (Berardi et al.,1995) and that of mouse (Tamai et al., 2000) and human (Ohashi et al., 2001) OCTN2 expressed in HEK293 cells. In Caco-2 cells (McCloud et al., 1996), however, L-carnitine uptake was not affected by the pH. The effect on L-carnitine uptake induced by changes in external pH was not due to an increase in transmembrane proton gradient, since the pH-induced stimulation was also seen in the absence of pH gradient (*see* Table 1). Depolarization of the membrane and/or the presence of an optimal conformation of the transporter might explain the apparent pH-dependent change of carnitine transport activity.

In conclusion, to our knowledge this is the first report showing Na^+/L-carnitine cotransport activity in both, enterocytes and BBMV. This transport system, which shows kinetics, substrate specificity and ion dependency similar to those of the Na^+/L-carnitine transporter OCTN2, must be the one mediating the

first step of intestinal L-carnitine absorption. In addition, the in situ hybridization studies revealed that chicken small intestine expresses OCTN2 mRNA.

The reason for the inability of previous studies to show the involvement of an active L-carnitine transport system in either in vivo (Gross & Henderson, 1984; Gudjonsson et al., 1985; Marciani et al., 1991; Gross & Savaiano 1993), in isolated enterocytes (Gross et al., 1986) or in intestinal BBMV (Li et al., 1990; Roque et al.,1996) is not clear. It does not appear to be a species-specific phenomenon since preliminary results revealed a Na^+/L-carnitine transport in rat small intestine. It could be related either to the presence of a significant unstirred water layer in the in vivo perfusion conditions, to the viability of the mammalian enterocyte preparations and to a significant L-carnitine binding component in BBMV preparations as compared with the actual transport, which could have overwhelmed the transport event.

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