Characterization of the endogenous carnitine transport and expression of a rat renal Na^+-dependent carnitine transport system in *Xenopus laevis* oocytes

Simona BERARDI,* Bruno HAGENBUCH,* Ernesto CARAFOLI† and Stephan KRÄHENBÜHL*‡

*Division of Clinical Pharmacology and Toxicology, Department of Internal Medicine, University Hospital Zürich, CH-8091 Zürich, Switzerland, and †Department of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zürich, Switzerland

L-Carnitine transport was characterized in *Xenopus laevis* oocytes before and after injection of mRNA isolated from rat renal cortex. Non-injected oocytes revealed endogenous Na^+-dependent transport of L-carnitine. After injection of 15 ng of rat kidney mRNA, the Na^+-dependent L-carnitine transport increased 2–3-fold, reaching maximal activity after 5–6 days. The expressed carnitine transport was maximal at pH 7.5, whereas the endogenous transport showed no clear maximum between pH 6.0 and 8.5. Kinetic analysis revealed apparent K_m values for L-carnitine of 66 μM for the endogenous and 149 μM for the expressed transport. Trimethyl-lysine and D-carnitine inhibited both the endogenous and the expressed transport. In contrast, L-acetylcarnitine, L-isovalerylcarnitine, L-palmitoylcarnitine and butyrobetaine inhibited predominantly the expressed transport, whereas glycinebetaine had no inhibitory effect on either transport system. Size-fractionated rat renal-cortex mRNA (median size 2 kb) induced a 3-fold higher L-carnitine transport than did unfractionated mRNA. These studies demonstrate that *Xenopus laevis* oocytes exhibit Na^+-dependent L-carnitine transport and provide the basis for expression-cloning of a rat renal Na^+-dependent L-carnitine transport system.

INTRODUCTION

Carnitine (4-*N*-trimethylammonio-3-hydroxybutanoate) is essential for the transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix across the mitochondrial inner membrane [1,2]. Another important role of carnitine is the so-called 'buffer function', which serves to maintain the cellular pool of free CoA [3,4]. By the reaction of carnitine with acyl-CoAs, which is mediated by carnitine acyltransferases, the respective acylcarnitines are formed, and, at the same time, free CoA is generated for intermediary metabolism [3–5]. In contrast with acyl-CoAs, acylcarnitines can be exported from the cells and can be eliminated by the kidneys [2].

The importance of carnitine in energy metabolism becomes evident in patients [6–8] and dogs [9] with primary carnitine deficiency. Under conditions where fatty acids become an important fuel, such as starvation and prolonged exercise, patients with primary carnitine deficiency can develop neurological symptoms and rhabdomyolysis [7,8]. The underlying defect in primary carnitine deficiency appears to be the decreased re-absorption of carnitine in the proximal tubule of the kidneys, leading to massive renal loss of carnitine, with decreased carnitine concentrations in plasma and tissues [7,8]. This defect is usually also expressed in fibroblasts and skeletal muscle [7,8], suggesting that these carnitine transport systems are identical. This suggestion is supported by the kinetic properties of these transport systems, showing a Na^+-dependent transport of L-carnitine and K_m values in the μmolar range for kidney, fibroblasts and skeletal muscle [2,10].

The renal L-carnitine transport system in brush-border membrane vesicles from rats was found to be Na^+-dependent with a K_m value of 55 μM, and could be partially inhibited by D-carnitine, acetylcarnitine and butyrobetaine, but not by trimethyl-lysine or choline [11]. The activity of this transport system decreased under a carnitine-supplemented diet, suggesting that the renal transport of L-carnitine is regulated by the carnitine concentration in plasma or urine.

So far, the molecular characterization of a L-carnitine transport system has not been published. As a first step in cloning an L-carnitine transport system, we characterized the endogenous transport of L-carnitine in *Xenopus laevis* oocytes and expressed and characterized a rat renal Na^+-dependent L-carnitine transporter.

MATERIALS AND METHODS

Materials

[^{3}H]Carnitine (79 Ci/mmol) was obtained from Amersham International (Aylesbury, Bucks., U.K). Guanidinium isothiocyanate, phenol and sucrose were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.), and PolyATtract mRNA isolation systems from Promega Corp. (Madison, WI, U.S.A.). Ethyl *m*-aminobenzoate (MS-222) was obtained from Sigma (St. Louis, MO, U.S.A.).

Isolation and fractionation of rat renal-cortex mRNA

Male Sprague–Dawley rats (final body weight approx. 200 g) were obtained from the Deutsche Versuchstiergesellgeschaft (Tuttlingen, Germany). After decapitation, both kidneys were removed, and the cortices were dissected and quickly homogenized with a Polytron (Kinematica G.m.b.H., Littau, Switzerland) in guanidinium isothiocyanate buffer [12]. Total RNA was

‡ To whom correspondence should be addressed.

isolated by phenol/chloroform extraction as described by Chomczynski and Sacchi [12]. From total RNA, mRNA was extracted by using the PolyATtract mRNA isolation system.

For size fractionation, mRNA samples (100 μg) were heated to 65 °C for 5 min and then loaded on a linear 6–30% (w/w) sucrose gradient containing 15 mM Pipes/NaOH, pH 6.4, 5 mM Na_2 EDTA and 0.25% Sarcosyl. The gradient was centrifuged at 4 °C for 35 min at 50000 rev./min (166667 *g*) in a Kontron TV 850 rotor. Thereafter, 23 1-ml fractions were collected from the bottom of the tube. Total and size-fractionated mRNA were precipitated with sodium acetate/ethanol [13], resuspended in water and stored at −80 °C. RNA concentrations were determined by measuring the absorption at 260 nm [13].

Expression of mRNA in *Xenopus laevis* oocytes

Mature *Xenopus laevis* females were purchased from H. Kähler (Hamburg, Germany) and kept under standard conditions [14]. Frogs were anaesthetized by immersion for 15 min in a 0.17% solution of ethyl *m*-aminobenzoate. Oocytes were collected as described [14] and incubated for 50 min at 19 °C in Ca^{2+}-free OR-2 solution [15] containing 2 mg/ml collagenase (Boehringer Mannheim, type D); this step was repeated, under microscopic inspection, until digestion was completed (usually another 50 min). Then the oocytes were washed in modified Barth's solution, containing (in mmol/l) 88 NaCl, 1 KCl, 2.3 $NaHCO_3$, 15 Hepes/NaOH, pH 7.6, 0.3 $Ca(NO_3)_2,4H_2O$, 0.41 $CaCl_2,6H_2O$, 0.82 $MgSO_4,7H_2O$, penicillin G (10 units/ml), streptomycin (10 μg/ml), gentamycin (10 μg/ml) and 50 mg/l type II-S soybean trypsin inhibitor (Sigma). After washing, stage-V and -VI oocytes were selected and incubated overnight at 18 °C in modified Barth's solution. The next day, healthy oocytes were injected with 50 nl of mRNA solution (usually containing 15 ng of mRNA), and cultured at 19 °C for 5 days (except where stated otherwise) with a daily change of modified Barth's solution.

Carnitine transport into oocytes

Cultured oocytes were washed once at room temperature in a Na^+-free buffer containing (in mmol/l) 100 choline chloride, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes/Tris, pH 7.5. Then 10–15 oocytes were incubated at 25 °C in 100 μl of transport medium, containing (in mmol/l) 100 NaCl or choline chloride, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes/Tris, pH 7.5, and 2 μCi of L-[^{3}H]carnitine at the concentration indicated in the Figure legends. L-Carnitine transport was terminated with 8 ml of ice-cold stop solution. The stop solution had a composition identical with that of the choline chloride transport medium, except that 1 mM L-carnitine was included, to decrease non-specific binding of the radioactive carnitine to oocytes. After three additional washes each with 8 ml of ice-cold stop solution, single oocytes were dissolved in 0.5 ml of 10% (w/w) SDS. To the resulting solution, 5 ml of scintillation fluid was added (Opti-Fluor, Packard), and radioactivity was counted in a Packard Tri-Carb 2200 CA liquid-scintillation analyser.

L-Carnitine transport kinetics into oocytes were fitted to Michaelis–Menten kinetics by using non-linear regression analysis (Sigmaplot Windows 1.0; Jandel Scientific, Erkrath, Germany).

RESULTS

The characterization of non-injected *Xenopus laevis* oocytes revealed endogenous Na^+-dependent L-carnitine transport, with a variable activity of approx. 1.50 pmol/h per oocyte at a carnitine concentration of 50 μM (see Table 1). Since this transport was not different for oocytes injected with 50 nl of water or not injected oocytes (results not shown), control oocytes were not injected in the experiments described below. After injection of 15 ng of mRNA isolated from rat renal cortex, L-carnitine transport into oocytes showed a linear increase with the incubation period over 5–6 days (results not shown). Therefore, an incubation period of 5 days was chosen between injection of mRNA and measurement of L-carnitine transport.

Table 1 Relationship between the amount of rat renal-cortex mRNA injected and Na^+-dependent L-carnitine transport into *Xenopus laevis* oocytes

L-Carnitine transport was measured for 1 h in the presence of a Na^+ gradient or in the presence of a choline gradient 5 days after injection of the oocytes. The L-carnitine concentration in the transport medium was 50 μM. Values are presented as means ± S.E.M. and represent the results obtained from 10–15 oocytes in one out of three experiments.

mRNA injected (μg/oocyte)	L-Carnitine uptake (pmol/h per oocyte)	
	Na^+ gradient	Choline gradient
0	2.63 ± 0.17	0.79 ± 0.03
5	4.78 ± 0.23	0.91 ± 0.05
10	6.60 ± 0.43	0.76 ± 0.03
15	5.83 ± 0.48	0.49 ± 0.02
25	5.72 ± 0.32	0.54 ± 0.03
35	5.53 ± 0.50	0.69 ± 0.07
50	5.88 ± 0.96	0.50 ± 0.06

As shown in Table 1, the increase in L-carnitine transport into oocytes after injection of different amounts of rat renal-cortex mRNA was dose-dependent, reaching a plateau at 10 ng of mRNA per oocyte. In comparison with non-injected oocytes, the Na^+-dependent transport of L-carnitine by oocytes injected with 10 ng of mRNA was increased by a factor of 2.5. In the experiments described below, 15 ng of rat renal-cortex mRNA was injected per oocyte.

The time-dependency of the L-carnitine transport into oocytes is shown in Figure 1. Na^+-dependent transport of L-carnitine increased with the incubation time for both injected and non-injected oocytes. The difference between injected and non-injected oocytes, presumably representing the expressed transport activity, was linear over the entire incubation time of 2 h. Based on these findings, an incubation time of 1 h was chosen for the following experiments.

Figure 2 illustrates that the endogenous and the expressed L-carnitine transport systems have different pH-dependencies. The expressed L-carnitine transport increased sharply between pH 7.0 and 7.5 and then remained constant up to pH 8.5. In contrast, the activity of the endogenous L-carnitine transport system increased over the entire pH range investigated, i.e. it did not exhibit a clear maximum.

The kinetic characterization of the endogenous and the expressed L-carnitine transport systems is shown in Figure 3. The curves relating the substrate concentration to the transport rate followed Michaelis–Menten kinetics in both injected and non-injected oocytes. In non-injected oocytes, Na^+-dependent L-carnitine transport reached a $V_{max.}$ of 5.3 pmol/h per oocyte (n = 2 experiments, range 4.9–5.7) and showed an apparent K_m value of 66 μM (range 54–78). On the other hand, the expressed L-carnitine transport activity (i.e. the difference between the Na^+-dependent L-carnitine transport into injected and non-injected

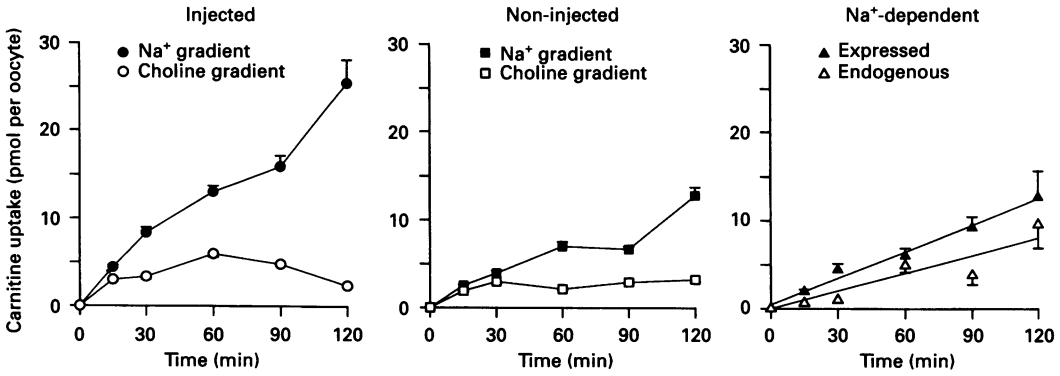

Figure 1 Time-dependency of the Na^+-dependent L-carnitine transport into *Xenopus laevis* oocytes

Rat renal-cortex mRNA (15 ng) was injected, and L-carnitine transport into injected or non-injected oocytes was measured 5 days after injection in the presence of a Na^+gradient or in the presence of a choline gradient. The L-carnitine concentration in the transport medium was 50 μM. Endogenous Na^+-dependent transport was calculated as the difference between total transport (that in the presence of a Na^+ gradient) and choline-dependent transport in non-injected oocytes. Expressed Na^+-dependent transport was calculated as the difference between total and choline-dependent transport in injected oocytes minus endogenous Na^+-dependent transport. Values are given as means ± S.E.M. and represent the results obtained for 10–15 oocytes in one out of three experiments.

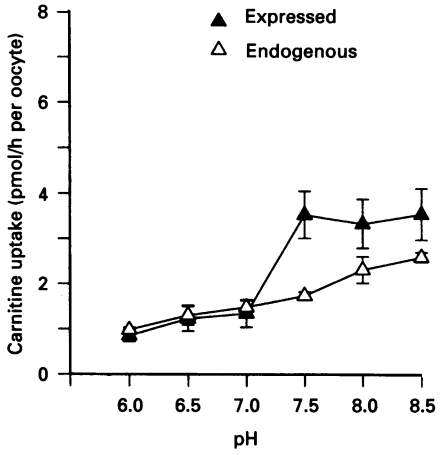

Figure 2 pH-dependency of the endogenous and the expressed Na^+-dependent L-carnitine transport

Rat renal-cortex mRNA (15 ng) was injected, and L-carnitine transport into injected or non-injected *Xenopus laevis* oocytes was measured for 1 h at 5 days after injection at the pH values indicated on the abscissa. The L-carnitine concentration in the transport media was 50 μM. Endogenous and expressed Na^+-dependent L-carnitine transport were calculated as described in Figure 1 legend. Values are given as means ± S.E.M. and represent the results obtained for 10–15 oocytes in one out of three experiments.

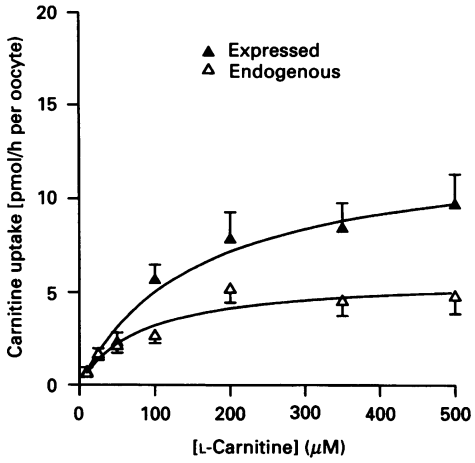

Figure 3 Kinetics of the Na^+-dependent L-carnitine transport into *Xenopus laevis* oocytes

Rat renal-cortex mRNA (15 ng) was injected, and L-carnitine transport into injected or non-injected oocytes was measured for 1 h at 5 days after injection in the presence of a Na^+ gradient or of a choline gradient at the L-carnitine concentrations indicated on the abscissa. Endogenous and expressed Na^+-dependent L-carnitine transport were calculated as described in Figure 1 legend. Both endogenous and expressed transport could be described by Michaelis–Menten kinetics. The calculated kinetic constants are given in the text. Values are expressed as means ± S.E.M. and represent the results for 10–15 oocytes in one out of two experiments.

oocytes) reached a $V_{max.}$ of 15.3 pmol/h per oocyte ($n = 2$ experiments, range 12.7–17.8) with an apparent K_m value of 149 μM (range 145–152). In contrast with the Na^+-dependent transport, the Na^+-independent transport failed to reach saturation up to an L-carnitine concentration of 500 μM (results not shown).

The inhibition of Na^+-dependent L-carnitine transport by intermediates of carnitine biosynthesis (trimethyl-lysine and butyrobetaine), acylcarnitines (L-acetylcarnitine, L-isovaleryl-carnitine, L-palmitoylcarnitine), D-carnitine or glycinebetaine is shown in Table 2. Whereas trimethyl-lysine and D-carnitine inhibited both the endogenous and the expressed Na^+-dependent L-carnitine transport, it is noteworthy that 5 μM L-palmitoyl-carnitine, 50 and 250 μM L-acetylcarnitine or L-isovaleryl-carnitine and 50 μM butyrobetaine inhibited predominantly the expressed L-carnitine transport. Higher concentrations of L-palmitoylcarnitine (50 μM) or butyrobetaine (250 μM) led to an increase in the Na^+-independent L-carnitine transport, suggesting impaired integrity of the oocytes (results not shown). Glycine-betaine, a quaternary amine containing two carbon atoms less than butyrobetaine, did not affect L-carnitine transport into oocytes up to a concentration of 250 μM.

Total renal-cortex mRNA was size-fractionated on a sucrose gradient and the fractions were injected into oocytes. As shown in Figure 4, the Na^+-dependent transport of L-carnitine into

Table 2 Inhibition of the Na^+-dependent L-carnitine transport into *Xenopus laevis* oocytes

Rat renal-cortex mRNA (15 ng) was injected into *Xenopus laevis* oocytes and L-carnitine transport into injected or non-injected oocytes was measured for 2 h with or without addition of inhibitors as indicated below. The L-carnitine concentration in the incubation medium was 50 μmol/l. The transport activities in the presence of a choline gradient were not influenced by the inhibitors used, and averaged 2.31 ± 0.18 pmol/2 h per oocyte for injected and 2.62 ± 0.23 pmol/2 h per oocyte for non-injected oocytes. Endogenous and expressed Na^+-dependent L-carnitine transport were calculated as described in Figure 1 legend. Data (pmol/2 h per oocyte) are given as means ± S.E.M. One value combines the data from three different experiments with 10–15 oocytes.

Inhibitor	Expressed transport	Inhibition (%)	Endogenous transport	Inhibition (%)
Control (no inhibitor)	12.3 ± 1.5	–	13.1 ± 0.9	–
Trimethyl-lysine (50 μM)	8.2 ± 1.0*	33.4	10.2 ± 0.7*	22.1
Trimethyl-lysine (250 μM)	0.5 ± 0.4*	95.9	6.0 ± 0.2*	54.4
L-Acetylcarnitine (50 μM)	9.4 ± 1.0	23.7	9.3 ± 0.8*	28.5
L-Acetylcarnitine (250 μM)	2.1 ± 0.7*	82.7	11.3 ± 0.7	13.8
D-Carnitine (50 μM)	9.8 ± 1.0	20.6	10.2 ± 0.6*	21.7
D-Carnitine (250 μM)	6.6 ± 0.6*	46.8	7.7 ± 0.3*	40.8
L-Palmitoylcarnitine (5 μM)	0.3 ± 1.2*	97.9	15.0 ± 1.1	–
Butyrobetaine (50 μM)	0.1 ± 0.7*	99.4	12.1 ± 0.7	7.2
L-Isovalerylcarnitine (50 μM)	3.5 ± 1.0*	71.8	19.8 ± 0.7*	–
L-Isovalerylcarnitine (250 μM)	1.3 ± 0.6*	89.1	14.7 ± 0.9	–
Glycinebetaine (50 μM)	10.8 ± 1.1	12.0	13.0 ± 0.6	0.4
Glycinebetaine (250 μM)	10.1 ± 1.1	17.9	11.3 ± 0.9	14.0

* $P < 0.05$ versus control values, by Student's t test.

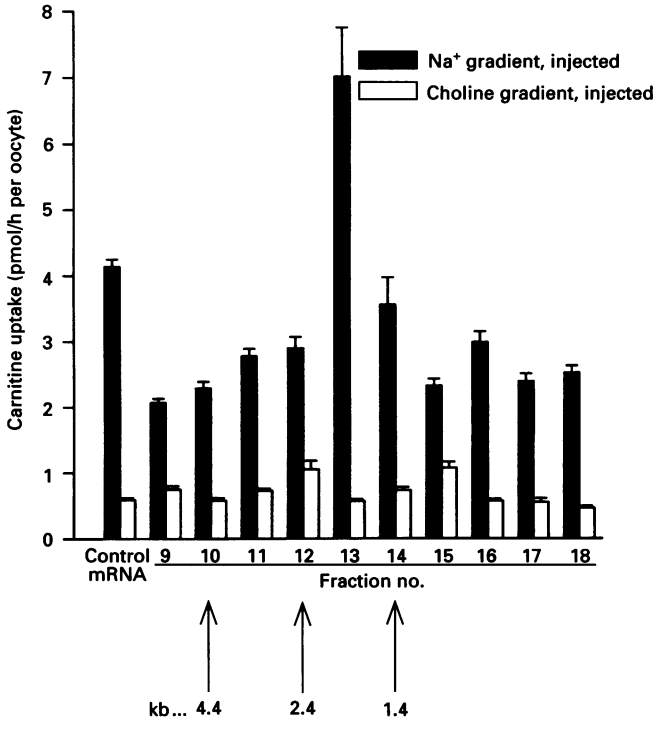

Figure 4 Fractionation of mRNA isolated from rat renal cortex on a linear sucrose density gradient

Rat renal-cortex mRNA (150 μg) was size-fractionated on a linear 6–20% (w/w) vertical sucrose gradient; 23 1-ml fractions were collected, and the mRNA from each fraction was precipitated, washed, and dissolved in water. From these fractions, a RNA gel was run, with molecular-size (kb) markers as indicated on the abscissa. Fractionated mRNA (15 ng contained in 50 nl) was injected into oocytes. After 5 days in culture, L-carnitine transport was determined in the presence of a Na^+ gradient or in the presence of a choline gradient. The values are presented as means ± S.E.M. and represent the results from 10–15 oocytes in one out of three experiments. Control values in non-injected oocytes were 1.88 ± 0.09 (Na^+ gradient) and 0.42 ± 0.03 (choline gradient) pmol/h per oocyte.

oocytes injected with fraction 13 (containing fragments of approx. 2 kb) was 3 times higher than in oocytes injected with unfractionated mRNA. Thus, fraction 13 was collected, and will be used for the construction of a cDNA library.

DISCUSSION

Concerning the important role of carnitine in energy metabolism [1–5], it was not surprising that *Xenopus laevis* oocytes exhibit an endogenous transport system for L-carnitine. In accord with the mammalian L-carnitine transport systems [2,10,11], the main driving force of L-carnitine transport into *Xenopus laevis* oocytes is a Na^+ gradient. The Na^+-dependent portion of L-carnitine transport in oocytes was saturable (Figure 3) and could be inhibited by carnitine analogues such as acylcarnitines, D-carnitine and intermediates of carnitine biosynthesis (Table 2). In contrast, the Na^+-independent portion of the endogenous L-carnitine transport was not saturable up to a carnitine concentration of 500 μM and could not be inhibited by carnitine analogues, findings which are compatible with a diffusion-mediated process. Although diffusion of carnitine has been described in rat jejunal brush-border membrane vesicles [16] and isolated perfused rat hearts [17], the existence of a low-affinity Na^+-independent L-carnitine transport system in oocytes cannot be excluded.

The endogenous and the expressed Na^+-dependent L-carnitine transport systems revealed different kinetic properties. As shown in Figure 3, the apparent K_m value of the latter was approx. 2 times higher than the corresponding value of the endogenous transport system. When compared with the K_m values obtained in rat renal brush-border membrane vesicles [11] or mouse kidney slices [18], the apparent K_m value of the expressed L-carnitine transport is 2–3 times higher. Since carnitine carriers with different kinetic properties may exist in mammals [10], the possibility cannot be excluded that more than one carnitine transport system was expressed in oocytes injected with rat kidney-cortex mRNA. Interestingly, similar differences have been reported between the K_m values of Na^+-dependent taurocholate

transport in rat liver sinusoidal-membrane vesicles and oocytes injected with total liver mRNA [19,20]. Although the exact reasons for these differences remain unclear, it should be noted that the K_m value in oocytes injected with cDNA coding for the Na^+-dependent rat liver sinusoidal taurocholate transporter was close to that obtained in sinusoidal-membrane vesicles [21], arguing against the possibility that the K_m value was affected by the composition of the oocyte plasma membrane.

Other dissimilarities between the endogenous and the expressed L-carnitine transport include differences in the pH-dependency (Figure 2) and in the inhibition pattern by carnitine analogues (Table 2). Taken together, these findings strongly suggest that the expressed L-carnitine transport reflects the expression of rat renal Na^+-dependent L-carnitine transport and does not represent stimulation of the endogenous carnitine transport system.

In contrast with other carnitine analogues, glycinebetaine, a quaternary amine structurally related to butyrobetaine, inhibited neither the endogenous nor the expressed Na^+-dependent transport (Table 2). This finding militates against the possibility that L-carnitine and glycinebetaine share the same transport system, and suggests different transport systems for quaternary amines in both *Xenopus laevis* oocytes and rat kidney. This is an important observation, since a Na^+-dependent glycinebetaine transport system has recently been characterized and cloned from rat renal cortex [22,23].

In conclusion, we have expressed and characterized an Na^+-dependent rat renal L-carnitine transport system in *Xenopus laevis* oocytes, whose kinetic properties are different from the endogenous Na^+-dependent transport of L-carnitine. These studies provide the basis for expression cloning of a rat renal Na^+-dependent L-carnitine transport system.

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