Archives of Biochemistry and Biophysics
Vol. 364, No. 1, April 1, pp. 99–106, 1999
Article ID abbi.1999.1118, available online at http://www.idealibrary.com on IDEAL®

3028

ND: 26159

Functional Characterization of the Carnitine Transporter Defective in Primary Carnitine Deficiency

Fernando Scaglia, Yuhuan Wang, and Nicola Longo[1]
Department of Pediatrics, Division of Medical Genetics, Emory University School of Medicine, Atlanta, Georgia, 30322

Received November 12, 1998, and in revised form January 12, 1999

Primary carnitine deficiency is an autosomal recessive disorder caused by defective carnitine transport which impairs fatty acid oxidation and manifests as nonketotic hypoglycemia or skeletal or heart myopathy. Here we report the functional characterization of this transporter in human fibroblasts. Carnitine enters normal cells by saturable and unsaturable routes, the latter corresponding to Na^+-independent uptake. Saturable carnitine transport was absent in cells from patients with primary carnitine deficiency. In control cells, saturable carnitine transport was energized by the electrochemical gradient of Na^+. Carnitine uptake was not inhibited by amino acid substrates of transport systems A, ASC, and X^-_{AG}, but was inhibited competitively (in potency order) by butyrobetaine > carnitine > palmitoylcarnitine = acetylcarnitine > betaine. Carnitine uptake was also noncompetitively inhibited by verapamil and quinidine, inhibitors of the multidrug resistance family of membrane transporters, suggesting that the carnitine transporter may share a functional motif with this class of transporters. A high-affinity carnitine transporter was present in kidney 293 cells, but not in HepG2 liver cells, whose carnitine transporter had a K_m in the millimolar range. These result indicate the presence of multiple types of carnitine transporters in human cells. © 1999 Academic Press

Key Words: **fatty acid oxidation; membrane transport; carnitine deficiency; human fibroblasts; membrane potential.**

Inherited disorders of fatty acid oxidation are characterized by inability to use fat as fuel during periods of stress and starvation with consequent nonketotic or hypoketotic hypoglycemia (1). Fatty acid oxidation defects can be divided into two groups: defects in the carnitine cycle and defects of the beta oxidation spiral (2). Carnitine is accumulated inside the cell by a specific membrane transporter and plays an essential role in fatty acid oxidation by binding long-chain fatty acids in the cytoplasm and transferring them to the mitochondrial matrix for subsequent oxidation.

Primary carnitine deficiency (OMIM No. 212140) is an autosomal recessive disorder caused by defective carnitine transport. Affected children present early in life with hypoketotic hypoglycemia or later in life with skeletal or heart myopathy. Plasma carnitine levels are markedly reduced (less than 10% of controls) without abnormal organic acids in the urine during acute attacks or excessive acylcarnitine, as observed in most cases of secondary carnitine deficiency (1). No evidence of defective carnitine biosynthesis or excessive carnitine degradation has been found in patients with systemic carnitine deficiency. Carnitine is lost in the urine due to impaired tubular resorption and fibroblasts share the defective transporter with the kidney (3, 4). This disorder responds promptly to dietary carnitine supplementation, with correction of metabolic, skeletal, and heart muscle abnormalities (1).

Analysis of carnitine transport in different tissues suggests the presence of heterogeneous transporters. Liver and brain have a low-affinity (K_m = 2–10 mM), high-capacity transporter (5–7), while the fibroblast, muscle, and heart cell have a high-affinity (K_m = 5–10 μM), low-capacity system (8, 9). Neuroblastoma cells have a transporter with intermediate affinity (100–200 μM) (10) as do muscle cells differentiated in culture (11). It is not known whether these different properties are due to different carnitine transporters, to the same transporter with different functional properties in different tissues, or to the contribution of less specific transporters to the total uptake in different tissues,

[1] To whom correspondence should be addressed at Department of Pediatrics, Division of Medical Genetics, Emory University, 2040 Ridgewood Dr., Atlanta, GA 30322. Fax: (404) 727 9398. E-mail: nl@rw.ped.emory.edu.

0003-9861/99 $30.00

resulting in an apparent lower affinity when nonspecific transporters predominate. In patients with primary carnitine deficiency, carnitine supplements restore promptly the content of carnitine in liver, but not in muscle, providing genetic evidence for at least two different carnitine transporters (1). To date, no cellular models have been reported for the liver or kidney carnitine transporter.

In this paper we functionally characterize the carnitine transporter defective in primary carnitine deficiency in cultured human fibroblasts. Our data define the relative power of different competitive inhibitors and identify verapamil and quinidine as novel noncompetitive inhibitors of this transporter. Our studies demonstrate quantitatively the dependence of this transporter on the sodium electrochemical potential and define new models in which to characterize liver and kidney carnitine transporters.

MATERIALS AND METHODS

Materials. DMEM (growth medium) was from Mediatech Inc. Fetal bovine serum was from HyClone. L-[methyl-^{3}H]Carnitine hydrochloride (85 Ci/mmol) and L-[2,3,4,5-^{3}H]arginine monohydrochloride (63 Ci/mmol) were from Amersham. 3-*O*-[methyl-^{3}H]Methyl-D-glucose (1000 mCi/mmol) was from Du Pont. Aldrich and Sigma were sources of unlabeled amino acids and other chemicals.

Cell culture and carnitine transport. Fibroblasts from patients 2996 and 10665 with primary carnitine deficiency were obtained as previously described (12). Both patients presented early in life (8 months and 2 years of age) with nonketotic hypoglycemia and Reye syndrome (12). Normal human fibroblasts were derived from adult skin biopsy as described previously (13). Human embryonic kidney 293 cells were obtained from Dr. Serena Bagnasco (Department of Pathology, Emory University). HepG2 cells were obtained from American Type Culture Collection. All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum. Cells were plated in 24-well plates (Costar) and grown to confluence. The medium was changed twice a week and 48 h before the transport experiment. Carnitine transport was measured with the cluster-tray method (14) in adherent cells with the same methods used to measure amino acid and glucose transport (13, 15). Briefly, on the day of the experiment cells were washed twice and preincubated for 90 min at 37°C in Tris (26 mM)-buffered (pH 7.4) Earle's balanced salt solution (EBSS) containing 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, which was always supplemented with 1% bovine serum albumin. This preincubation was performed to deplete the intracellular amino acid pool as well as the intracellular stores of carnitine and to minimize possible trans-effects such as those observed in several amino acid transport systems (16). Bovine serum albumin was included in the solution to avoid perturbation of Na^+ and K^+ gradients observed in human fibroblasts incubated in the absence of serum (17). Cells were then incubated for 2 h in the same medium containing 0.5 μM carnitine (0.5 μCi/ml). After transport assay, cell monolayers were rapidly washed three times with ice-cold 0.1 M $MgCl_2$ (total washing time below 10 s). Ethanol (0.5 ml) was then added to each well to extract intracellular radioactivity, mixed with 4 ml of scintillation fluid, and counted in a beta counter (Beckman LS 7500). Cell monolayers were dried, solubilized with 200 μl of 0.1% sodium deoxycholate in 1 M NaOH, and assayed for protein using a modified Lowry procedure (18). Intracellular water was evaluated in parallel experiments from the equilibrium distribution of 3-*O*-methyl-D-glucose (19), which is reached within 10 min in cultured human fibroblasts (13).

Initial experiments indicated that carnitine uptake was linear up to 4 h (12) and a transport time of 2 h was chosen for subsequent experiments to assess initial rates of entry of this solute.

Calculations. Carnitine influx was normalized for cell protein (measured in the same well) and cell water (measured in a parallel tray) and expressed as nmol * ml cell water^{-1} * h^{-1}. Calculations were performed using a BASIC program on an IBM computer. Data from three independent wells within the same cluster trays were averaged and data are expressed as means ± SD of triplicates, unless otherwise indicated. Determination of kinetic parameters for carnitine transport was performed by nonlinear regression analysis (after correction for nonsaturable uptake measured with 2 mM cold carnitine) according to a Michaelis–Menten equation.

When correction for unsaturable uptake was not possible, the following equation was used for a saturable system plus diffusion,

$$v = \frac{V^*_{max}[S]}{K_m + [S]} + K^*_d[S], \quad [1]$$

where v is the observed velocity of substrate entry, $[S]$ is the substrate concentration, V_{max} is the maximal velocity, K_m is the concentration of substrate at which half-maximal velocity is observed, and K_d is the diffusion constant (13).

For inhibition studies, kinetic analysis was performed in the presence of fixed concentrations of inhibitor to determine if inhibition was competitive or noncompetitive. To determine the relative power, carnitine transport was measured in the presence of increasing concentrations of inhibitor and data fitted to the equation

$$v = V_0 - \frac{I^*_{max}[I]}{I_{0.5} + [I]}, \quad [2]$$

where V_0 is the transport velocity in the absence of the inhibitor, I_{max} is the maximal inhibition, $[I]$ is the inhibitor concentration, and $I_{0.5}$ is the concentration of inhibitor at which half-maximal inhibition is achieved (13).

The Hill equation was used to estimate the minimum number (n) of sodium binding sites on the transporter. Nonlinear parameters are reported in the text as means ± SD.

Membrane potential was calculated from the distribution ratio of L-arginine (20). This cationic amino acid is transported by human fibroblasts through transport system y^+ and its equilibrium distribution depends only on membrane potential (20). Membrane potential was calculated by applying the Nernst equation to the distribution ratio of L-arginine $[Arg]_{in}/[Arg]_{out}$ (20).

Intracellular ion concentrations were determined by flame photometry as previously described (21, 22).

RESULTS

Carnitine transport in human fibroblasts. Carnitine (0.5 μM) transport in fibroblasts from controls and patient 2996 with primary carnitine deficiency is reported in Fig. 1A in the presence or absence of sodium and excess unlabeled carnitine (2 mM). In normal cells, most carnitine transport was Na^+ dependent, with the saturable component (i.e., the component inhibitable by 2 mM cold carnitine) accounting for 80–92% of total in different experiments (Fig. 1A). The nonsaturable component was not significantly different from carnitine uptake in the absence of sodium. Na^+-independent

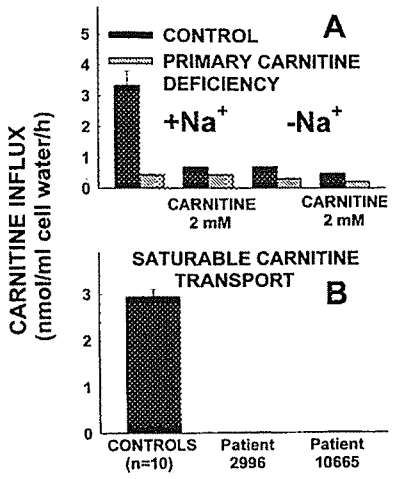

FIG. 1. Carnitine transport in fibroblasts from controls and patients with primary carnitine deficiency. (A) Effect of extracellular sodium. Carnitine (0.5 μM) transport was measured for 2 h in fibroblasts from controls and patient 2996 with primary carnitine deficiency in the presence (left) and absence (right) of sodium (117 mM) and carnitine (2 mM). Data are means ± SD of triplicates. (B) Saturable carnitine transport. Carnitine (0.5 μM) was measured as above. Carnitine uptake in the presence of 2 mM carnitine was subtracted from total uptake to obtain saturable carnitine transport. Points are means ± SE of six observations in patients' cells and 36 observations in 10 different control strains. Saturable carnitine transport was significantly ($P < 0.01$) reduced in cells from both patients with primary carnitine deficiency and was not significantly different from zero.

carnitine transport was only minimally inhibited by the addition of unlabeled carnitine, confirming that the nonsaturable component corresponded to Na^+-independent uptake. By contrast, carnitine transport in the absence or presence of excess carnitine was identical in cells from patient 2996 with primary carnitine deficiency, indicating that the saturable, sodium-dependent component was lacking in these cells. When total carnitine uptake was corrected for residual uptake in the presence of 2 mM carnitine to obtain saturable carnitine transport, cells from two patients with primary carnitine deficiency had absent carnitine transport (Fig. 1B).

Energization of the carnitine transporter. We tested the dependence of carnitine transport on the electrochemical potential of Na^+ in human fibroblasts. Carnitine transport was measured at three different carnitine concentrations (0.5, 2.5, and 10 μM) in the presence of increasing extracellular concentrations of Na^+ (0–140 mM). Na^+ was substituted by methylglucamine and chloride remained constant at all concentrations. Carnitine transport was strongly dependent on extracellular Na^+ concentration (Fig. 2). Maximal carnitine influx was observed at approximately 50 mM Na^+. Half-maximal carnitine influx was observed at 11.4 ± 2.1, 6.6 ± 1.3, and 4.0 ± 0.9 mM when carnitine concentrations were 0.5, 2.5, and 10 μM, respectively. When data of Fig. 2 were fitted to a Hill equation, the n obtained were 1.21 ± 0.25, 0.92 ± 0.26, and 0.87 ± 0.35 at 0.5, 2,5, and 10 μM carnitine, respectively. In all cases, the n obtained was not significantly different from 1, indicating that the transport of carnitine did not require cooperative interactions and suggesting that one sodium ion was cotransported with each molecule of carnitine.

Membrane potential of human fibroblasts was varied using increasing potassium concentrations (5.4–105.4 mM) in the extracellular medium, while the concentration of Na^+ was kept constant (50 mM). Methylglucamine chloride was exchanged for KCl. The L-arginine distribution ratio was used to measure membrane potential, since the accumulation of this amino acid through system y^+ depends solely on membrane potential and no significant metabolism occurs during the 60 min required to achieve steady-state levels (20). As expected, the L-arginine distribution ratio decreased with increasing extracellular potassium concentration (Fig. 3A). The membrane potential, estimated by applying the Nernst equation to the distribution ratio of L-arginine (20), decreased from −63.8 ± 1.0 mV at 5.4 mM to −37.7 ± 2.3 mV at 105.4 mM extracellular $[K^+]$. Carnitine transport also decreased with increasing concentrations of potassium (Fig. 3B), showing a linear dependence on membrane potential (Fig. 3C).

Ouabain is an inhibitor of Na,K-ATPase which increases intracellular $[Na^+]$ and decreases membrane potential in human fibroblasts (21). We tested the effect of the cardiac glycoside on carnitine transport, intracellular ion concentrations, and membrane potential. The membrane potential of human fibroblasts incubated in the absence of ouabain was −66.5 ± 0.1 mV

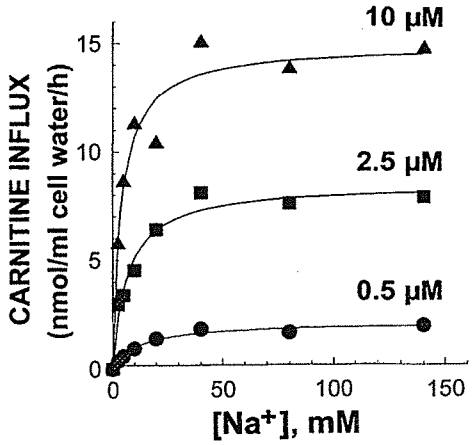

FIG. 2. Dependence of carnitine transport on chemical potential of Na^+ in human fibroblasts. Carnitine (0.5, 2.5, and 10 μM) transport was measured for 2 h in the presence of increasing extracellular concentrations of Na^+ (0–140 mM). Data are means ± SD of triplicates. Lines represent best fits of data to a Hill equation.

* Se non influenzano il Vmax, e il loro Vmax è uguale alla Vmax di arginina che utilizzano lo stesso trasportatore -

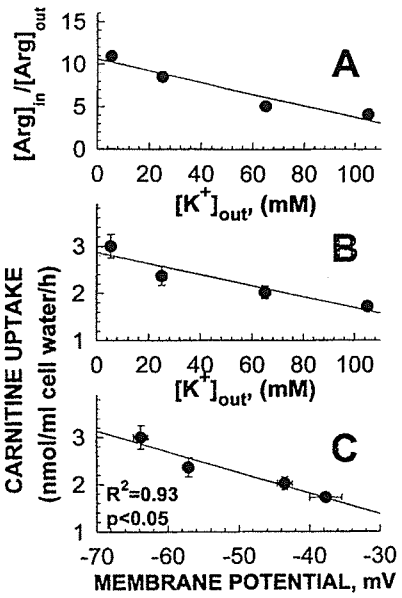

FIG. 3. Dependence of carnitine transport on membrane potential. Carnitine (0.5 μM, B) transport and arginine (20 μM) distribution ratio (A) were measured for 2 h in the presence of increasing concentrations of extracellular K^+ (5.4–105.4 mM). Data are means ± SD of triplicates. Membrane potential was estimated from the distribution ratio of arginine and C depicts carnitine influx as a function of membrane potential. Regression in C was statistically significant ($P < 0.05$) using analysis of variance.

and decreased to −56.5 ± 0.2 mV after 2 h incubation with 0.4 mM ouabain. Intracellular [Na^+] increased from 16.7 ± 1.7 to 78.2 ± 7.5 mM, while intracellular [K^+] decreased from 186.2 ± 7.3 to 145.7 ± 12.8 mM. Since extracellular [Na^+] was constant at 124 mM, the Na^+ electrochemical potential (calculated as in Ref. 23) decreased from 11.58 to 6.63 kJ * mol^{-1}. Saturable carnitine transport decreased from 2.45 ± 0.32 to 1.33 ± 0.24 nmol * ml cell $water^{-1}$ * h^{-1} with 0.4 mM ouabain. The ratio between carnitine transport and Na^+ electrochemical potential remained similar in cells incubated with or without ouabain (from 0.21 ± 0.03 to 0.20 ± 0.04 nmol of carnitine * ml of intracellular $water^{-1}$ * h^{-1} per kJ * mol^{-1} of Na^+ electrochemical potential without and with ouabain, respectively), indicating that the effect of ouabain was fully explained by its alteration of the Na^+ electrochemical potential gradient.

Characterization of sodium-dependent carnitine transport. Na^+-dependent carnitine transport by human fibroblasts was not inhibited by amino acid substrates of the Na^+-dependent systems A (L-proline and methylaminoisobutyric acid), ASC (L-alanine and L-threonine), and X^-_{AG} (L-aspartate and L-glutamate) (Table I). Creatine, taurine, GABA, choline, phloretin, phloridzin, dimethyl glycine, sarcosine, cyclosporin A, tolbutamide, and trimethyl lysine, one of the precursors in the synthesis of carnitine, also failed to inhibit carnitine uptake. By contrast, acetylcarnitine, betaine, butyrobetaine, palmitoylcarnitine, quinidine, and verapamil caused a marked inhibition of carnitine transport.

To define if these compounds inhibited carnitine transport by a competitive or noncompetitive mechanism, kinetics of carnitine influx were measured in the presence of fixed concentrations of inhibitors (Fig. 4). Butyrobetaine, acetylcarnitine, palmitoylcarnitine, and betaine increased the K_m for carnitine transport, affecting only minimally the V_{max}. By contrast, verapamil and quinidine decreased mostly the V_{max} for carnitine uptake, without affecting greatly the K_m (Table II). These results indicated that butyrobetaine, acetylcarnitine, palmitoylcarnitine, and betaine were competitive inhibitors, while quinidine and verapamil were noncompetitive inhibitors.

Measurement of carnitine uptake in the presence of increasing concentrations of inhibitors indicated that carnitine transport was inhibited, in potency order, by butyrobetaine ($I_{0.5}$ = 3.9 ± 0.3 μM) > carnitine ($I_{0.5}$ = 7.2 ± 0.6 μM) > palmitoylcarnitine ($I_{0.5}$ = 13.1 ± 3.0 μM) = acetylcarnitine ($I_{0.5}$ = 14.2 ± 1.3 μM) > betaine ($I_{0.5}$ = 187.7 ± 25.0 μM) (Fig. 5).

Similar experiments with verapamil and quinidine indicated that the noncompetitive inhibitors half-maximally inhibited carnitine transport at 53 ± 10 μM (verapamil) and 100 ± 40 μM (quinidine) (not shown).

Carnitine transport in liver and kidney cells. Several studies have examined carnitine transport in liver and kidney. Isolated rat liver cells accumulate carnitine by a low-affinity transporter with a K_m of around 5 mM (6) which is comparable to the K_m of 2.6–4.2 mM measured in the perfused organ (7). A high-affinity transporter for carnitine (K_m = 90 μM) has been proposed in rat kidney cortex slices (24). Studies in rat kidney brush border vesicles indicated the presence of two transporters, one with high affinity (K_m = 17 μM) and one with low affinity (K_m = 15 mM) (25). Kidney studies are complicated by the presence of different types of cells in the same preparations and by the use of different techniques. There are no reports of carnitine transport in cells in secondary culture obtained from these organs. We measured carnitine transport in human liver HepG2 cells and human embryonal kidney 293 cells. Initial experiments at low concentrations of carnitine indicated the absence of a high-affinity component in liver HepG2 cells after correction for unsaturable uptake measured in the presence of 2 mM unlabeled carnitine. At higher carnitine concentrations (0.1–50 mM), HepG2 cells had a low-affinity (K_m = 2.0 ± 0.9 mM) carnitine transporter in addition to a linear component formally indistinguishable from diffusion (Figs. 6A and 6B). By contrast, when total car-

TABLE I

Inhibition of Carnitine Transport

Inhibitor	Concentration (mM)	Transport (nmol/ml cell water/h)	% of control
None	—	3.95 ± 0.50	100
Carnitine-related compounds			
Acetylcarnitine	2	0.49 ± 0.03*	12
Betaine	2	0.53 ± 0.03*	13
Butyrobetaine	2	0.36 ± 0.06*	9
L-Carnitine	2	0.40 ± 0.04*	10
Choline	2	3.14 ± 0.12	79
Creatine	1	3.53 ± 0.36	89
Dimethyl glycine	2	4.57 ± 0.74	116
Palmitoylcarnitine	1	0.79 ± 0.24*	20
Sarcosine	2	3.47 ± 0.69	88
Trimethyl lysine	2	3.21 ± 0.20	81
Amino acids			
L-Alanine	2	3.70 ± 0.04	94
L-Arginine	2	3.16 ± 0.17	80
GABA	2	3.12 ± 0.12	79
L-Glutamic acid	2	3.74 ± 0.53	95
L-Histidine	2	3.15 ± 0.03	80
L-Leucine	1	2.98 ± 0.12	75
L-Proline	2	3.96 ± 0.28	100
L-Taurine	2	3.30 ± 0.02	84
L-Threonine	2	3.61 ± 0.10	91
MeAIB	2	3.83 ± 0.38	97
Others			
Cyclosporin A	0.1	3.19 ± 0.21	81
Phloretin	0.5	3.84 ± 0.12	97
Phloridzin	0.5	3.75 ± 0.10	95
Quinidine	0.5	0.76 ± 0.01*	19
Tolbutamide	0.5	3.20 ± 0.10	81
Verapamil	0.5	0.31 ± 0.01*	8

Note. Carnitine (0.5 μM) uptake by human fibroblasts was measured for 2 h in the presence of the indicated concentrations of inhibitors. Points are means ± SD of triplicates (12 observations for no inhibitor).

* $P < 0.01$ versus basal (no inhibitor).

nitine uptake was corrected for unsaturable uptake (measured with 2 mM cold carnitine), kidney 293 cells transported carnitine with a high-affinity (K_m = 4.2 ± 0.9 μM) carrier similar to that of human fibroblasts (Figs. 6C and 6D).

DISCUSSION

Carnitine plays an essential role in fatty acid oxidation and its deficiency results in defective energy production during starvation with fasting hypoglycemia and heart and skeletal myopathy (1). In this paper, we functionally characterize the carnitine transporter defective in the autosomal recessive disorder primary carnitine deficiency. This transporter is expressed both in the kidney and in cultured fibroblasts. Normal control fibroblasts transported carnitine by a saturable and an unsaturable route, indistinguishable from Na^+-independent transport (Fig. 1). The latter component could be subtracted from total uptake to obtain the saturable component, which was a high-affinity (K_m = 7.0 ± 0.4 μM), low-capacity Na^+-dependent transport system (Table II).

Cells from two patients with primary carnitine deficiency lacked mediated carnitine transport and only had Na^+-independent, unsaturable carnitine uptake (Fig. 1), providing genetic evidence that the high-affinity Na^+-dependent carnitine transporter of normal fibroblasts is the one defective in primary carnitine deficiency.

The carnitine transporter of human fibroblasts was energized by the Na^+ electrochemical potential gradient (Figs. 2 and 3). At three different extracellular carnitine concentrations, there was a saturable increase in carnitine uptake with increasing Na^+ concentration. Nonlinear regression analysis of these curves according to a Hill equation generated an n around 1, indicating that carnitine transport does not involve cooperative interactions and suggesting that each molecule of carnitine is cotransported with a single Na^+. An increase in extracellular potassium concentrations

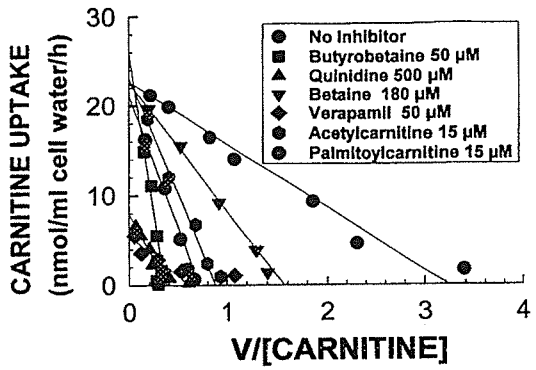

FIG. 4. Kinetics of carnitine uptake in the presence of fixed concentrations of inhibitors. Carnitine (0.5–100 μM) transport was measured for 2 h in the presence of the indicated concentrations of inhibitors. Data were corrected for diffusion (measured in the presence of 10 mM cold carnitine) and reported in an Eadie–Hofstee plot. Lines represent best fits of data to a Michaelis–Menten equation.

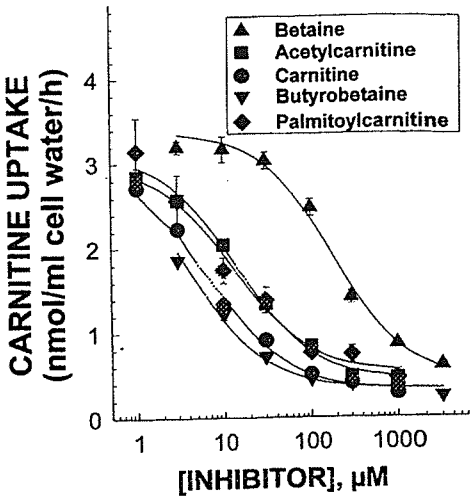

FIG. 5. Inhibition of carnitine transport by betaine, acetylcarnitine, carnitine, butyrobetaine, and palmitoylcarnitine. Carnitine (0.5 μM) transport was measured for 2 h in the presence of indicated concentrations of inhibitors. Points are means ± SD of triplicates. Data were fit to Eq. [2] (see Materials and Methods) to determine the concentration of inhibitor producing half-maximal inhibition and the best fit of data is shown.

decreased membrane potential and carnitine transport. Carnitine transport was linearly related to the variation in membrane potential (Fig. 3). Additionally, incubation of human fibroblasts in the presence of ouabain reduced carnitine transport in direct correlation with the decreased Na^+ electrochemical potential gradient due to both increased intracellular [Na^+] and reduced transmembrane electrical potential. These results indicate that carnitine transport in human fibroblasts depends on the Na^+ electrochemical potential. Previous studies in human fibroblasts have shown that the requirement for Na^+ is only partially fulfilled by substitution with Li^+, and not with Rb^+ or K^+, and that incubation in the presence of Na^+ ionophors or metabolic inhibitors (rotenone, antimycin A, and potassium cyanide) decreased carnitine accumulation (26). The mechanism causing such effects has not been defined and our results suggest that at least part of these effects may be due to alterations in either the transmembrane electrical potential or the Na^+ chemical gradient, which were not measured in previous studies.

TABLE II

Kinetic Constants for Carnitine Transport in the Presence of Fixed Concentrations of Inhibitors

	V_{max} (nmol/ml cell water/h)	K_m (μM)
No inhibitor	22.7 ± 0.4	7.0 ± 0.4
Acetylcarnitine (15 μM)	23.2 ± 0.6	26.3 ± 1.9
Betaine (180 μM)	22.6 ± 0.2	14.4 ± 0.5
Butyrobetaine (50 μM)	26.0 ± 1.5	72.3 ± 0.8
Palmitoylcarnitine (15 μM)	21.2 ± 0.4	30.1 ± 1.6
Quinidine (500 μM)	7.9 ± 0.3	20.6 ± 1.9
Verapamil (50 μM)	5.5 ± 0.7	9.2 ± 4.0

Note. Confluent cells were incubated for 2 h with increasing concentrations of carnitine (1–2000 μM) in the absence or presence of the indicated concentrations of inhibitors. Data were corrected for unsaturable uptake (measured in the presence of 2 mM cold carnitine) and analyzed by nonlinear regression analysis according to a Michaelis–Menten equation. Values represent best fits of data ± 95% confidence intervals. The best fit is reported as a straight line in the Eadie–Hofstee plots shown in Fig. 4.

Carnitine transport was not inhibited by any of the amino acids substrates of the Na^+-dependent transport systems active in human fibroblasts: systems A, ASC, and X^-_{AG} (15, 27) (Table I). By contrast, significant inhibition could be obtained by acetylcarnitine, betaine, butyrobetaine, and palmitoylcarnitine in addition to quinidine and verapamil.

Inhibition by acetylcarnitine, betaine, butyrobetaine, and palmitoylcarnitine was competitive while that from quinidine and verapamil was of the noncompetitive type (Fig. 4 and Table II). The relative power of competitive inhibitors was further characterized by determining the concentrations needed to cause half-maximal inhibition of carnitine transport (Fig. 5). With this type of analysis, butyrobetaine was even more effective than carnitine in inhibiting the carnitine transporter, the acylcarnitines palmitoylcarnitine and acetylcarnitine had about equal power, while betaine was the less powerful agent.

All competitive inhibitors share with carnitine the presence of a trimethylamino group on one end and a carboxyl group on the other side. Inhibition could not be seen with trimethyl lysine, which has an additional amino group close to the carboxyl group, with choline (at relatively low concentrations), which has a CH_2OH

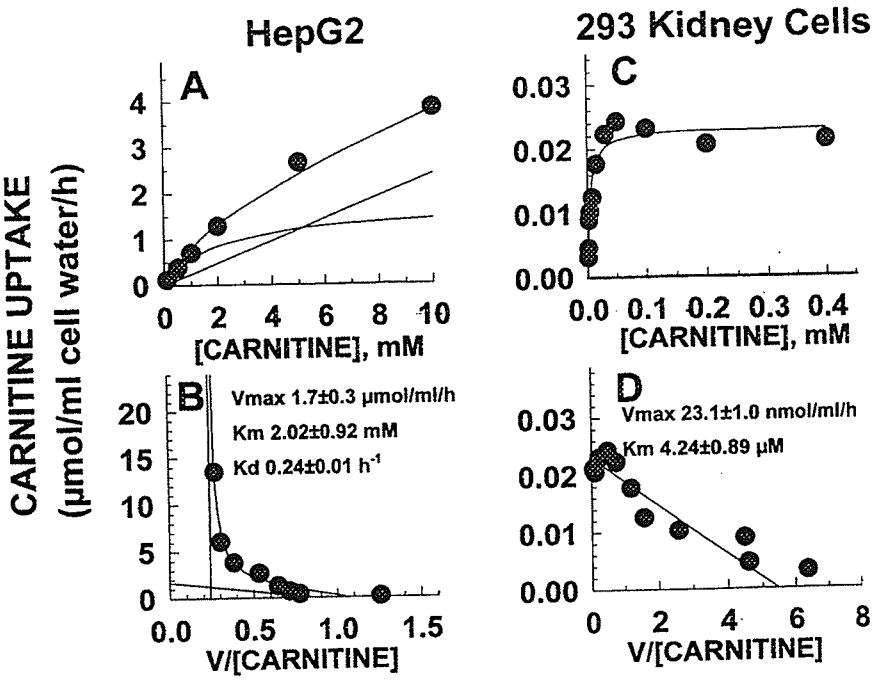

FIG. 6. Kinetics of carnitine transport in human hepatoma HepG2 (A, B) and human 293 kidney (C, D) cells. Carnitine transport was measured for 2 h. Diffusion was estimated by computer in the case of HepG2 cells, since about 40% of carnitine transport at 10 mM was due to a mediated component (see individual components in A). By contrast, in kidney cells as in fibroblasts, mediated transport was only a minimal fraction (<2%) of total carnitine transport at 10 mM and diffusion could be subtracted prior to plotting. Data are means of triplicates and were analyzed by nonlinear regression analysis using Eq. [1] (HepG2) or a simple Michaelis–Menten equation for 293 cells. Lines represent best fits to data. Data are shown according to an Eadie–Hofstee plot in B and D.

group instead of a carboxyl group, or with dimethyl glycine, which has a dimethyl, rather than a trimethyl amino group.

Even though betaine was the less powerful competitive inhibitor, its dose required for half-maximal inhibition of carnitine transport (188 ± 25 µM) was actually lower than that required to block GABA entry through the human betaine transporter (250–500 µM, Ref. 28). This suggested that the carnitine transporter may share some recognition properties with the betaine transporter. The human betaine transporter is noncompetitively inhibited by verapamil and quinidine (28). Verapamil and quinidine also inhibited carnitine transport (Table II) with a noncompetitive mechanism (Fig. 4). Verapamil and quinidine are inhibitors of the ABC family of membrane transporters, which include the multidrug resistance family of proteins (29). Half-maximal inhibition of carnitine transport by these compounds occurred at concentrations similar to those reported for the inhibition of the multidrug resistance pump (50–100 µM, Refs. 30 and 31). However, the carnitine transporter is driven by the Na^+ electrochemical gradient (Figs. 2 and 3) and cyclosporin A, a well-known inhibitor of the multi-drug-resistant protein (29), had no effect on carnitine transport (Table I). It is possible that the carnitine transporter contains a structural motif related to the multi-drug-resistant protein explaining the inhibition by verapamil and quinidine.

Human embryonal kidney 293 cells expressed a high-affinity carnitine transporter similar to that defective in primary carnitine deficiency (Fig. 6). By contrast, human hepatoma HepG2 cells transported carnitine with a low-affinity transporter whose K_m (2 mM) was three orders of magnitude greater than that of human fibroblasts. These data are consistent with *in vivo* studies of patients with primary carnitine deficiency, who waste carnitine in the urine (1). In these patients, carnitine therapy restores carnitine content in the liver, but not in the muscle (1), indicating that the transporter defective in primary carnitine deficiency does not impair carnitine transport by the liver. Studies in animal tissues or isolated cells have confirmed that the liver has a low-affinity carnitine transporter (6, 7). Our studies indicate that human HepG2 liver cells express this low-affinity carnitine transporter and can be used to further characterize its features.

While this paper was in preparation, a putative carnitine transporter (OCTN2), initially identified as an organic cation transporter in a human trophoblast cell line (32), was cloned from a human kidney cDNA li-

brary (33). The functional properties of the cDNA expressed in 293 cells strictly correspond to those described here in human fibroblasts, rendering the OCTN2 gene an excellent candidate for primary carnitine deficiency (33). Interestingly, the OCTN2 cDNA shares a nucleotide binding fold motif with ABC transporters (33). This domain may be responsible for the unusual inhibition by verapamil and quinidine described in this paper.

REFERENCES

1. Roe, C. R., and Coates, P. M. (1995) *in* The Metabolic and Molecular Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.), pp. 1501–1533, McGraw-Hill, New York.
2. Pons, R., and De Vivo, D. C. (1995) *J. Child Neurol.* **10**(Suppl. 2), 2S8–2S24.
3. Eriksson, B. O., Gustafson, B., Lindstedt, S., and Nordin, I. (1989) *J. Inher. Metab. Dis.* **12,** 108–111.
4. Treem, W. R., Stanley, C. A., Finegold, D. N., Hale, D. E., and Coates, P. M. (1988) *New Engl. J. Med.* **319,** 1331–1336.
5. Burlina, A. P., Sershen, H., Debler, E. A., and Latjha, A. (1989) *Neurochem. Res.* **14,** 489–493.
6. Christiansen, R. Z., and Bremer, J. (1976) *Biochim. Biophys. Acta* **448,** 562–577.
7. Kispal, G., Melegh, B., Alkonyi, I., and Sandor, A. (1987) *Biochim. Biophys. Acta* **896,** 96–102.
8. Bohmer, T., Eiklid, K., and Jonsen, J. (1977) *Biochim. Biophys. Acta* **465,** 627–633.
9. Rebouche, C. J., and Engel A. G. (1982) *In Vitro* **18,** 495–500.
10. Nalecz, K. A., Korzon, D., Wawrzenczyk, A., and Nalecz, M. J. (1995) *Arch. Biochem. Biophys.* **322,** 214–220.
11. Martinuzzi, A., Vergani, L., Rosa, M., and Angelini, C. (1991) *Biochim. Biophys. Acta* **1095,** 217–222.
12. Scaglia, F., Wang, Y., Singh, R. H., Dembure, P. P., Pasquali, M., Fernhoff, P. M., and Longo, N. (1998) *Genet. Med.* **1,** 34–39.
13. Longo, N., Griffin, L. D., and Elsas, L. J. (1988) *Am. J. Physiol.* **254,** C628–C633.
14. Gazzola, G. C., Dall'Asta, V., Franchi-Gazzola, R., and White, M. F. (1981) *Anal. Biochem.* **115,** 368–374.
15. Longo, N., Franchi-Gazzola, R., Bussolati, O., Dall'Asta, V., Foa, P. P., Guidotti, G. G., and Gazzola, G. C. (1985) *Biochim. Biophys. Acta* **844,** 216–223.
16. Gazzola, G. C., Dall'Asta, V., and Guidotti, G. G. (1980) *J. Biol. Chem.* **255,** 929–936.
17. Dall'Asta, V., Gazzola, G. C., Longo, N., Bussolati, O., Franchi-Gazzola, R., and Guidotti, G. G. (1986) *Biochim. Biophys. Acta* **860,** 1–8.
18. Wang, C. S., and Smith, R. L. (1975) *Anal. Biochem.* **63,** 414–417.
19. Kletzien, R. F., Pariza, M. W., Becker, J. E., and Potter, V. R. (1975) *Anal. Biochem.* **68,** 537–544.
20. Bussolati, O., Laris, P. C., Nucci, F. A., Dall'Asta, V., Longo, N., Guidotti, G. G., and Gazzola, G. C. (1987) *Am. J Physiol.* **253,** C391–C397.
21. Longo, N., Griffin, L. D., and Elsas, L. J. (1991) *Am. J. Physiol.* **260,** C1341–C1346.
22. Longo, N. (1996) *Biochim. Biophys. Acta* **1381,** 28–44.
23. Dall'Asta, V., Bussolati, O., Guidotti, G. G., and Gazzola, G. C. (1991) *J. Biol. Chem.* **266,** 1591–1596.
24. Huth, P. J., and Shug, A. L. (1980) *Biochim. Biophys. Acta* **602,** 621–634.
25. Stieger, B., O'Neill, B., and Krahenbuhl, S. (1995) *Biochem. J.* **309,** 643–647.
26. Tein, I., Bukovac, S. W., and Xie, Z-W. (1996) *Arch. Biochem. Biophys.* **329,** 145–155.
27. Dall'Asta, V., Gazzola, G. C., Franchi-Gazzola, R., Bussolati, O., Longo, N., and Guidotti, G. G. (1983) *J. Biol. Chem.* **258,** 6371–6379.
28. Rasola, A., Galietta, L. J. V., Barone, V., Romeo, G., and Bagnasco, S. (1995) *FEBS Lett.* **373,** 229–233.
29. Sonneveld, P., and Wiemer, E. (1997) *Curr. Opin. Oncol.* **9,** 543–548.
30. Ayesh, S., Shao, Y-M., and Stein, W. D. (1996) *Biochim. Biophys. Acta* **1316,** 8–18.
31. Eliason, J. F., Ramuz, H., and Kaufmann, F. (1990) *Int. J. Cancer* **46,** 113–117.
32. Wu, X., Prasad, P. D., Leibach, F. H., and Ganapathy, V. (1998) *Biochem. Biophys. Res. Commun.* **246,** 589–595.
33. Tamai, I., Oshashi, R., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., Sai, Y., and Tsuji, A. (1998) *J. Biol. Chem.* **273,** 20378–20382.