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Reconstitution into liposomes and functional characterization of the carnitine transporter from renal cell plasma membrane

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Abstract

The carnitine transporter was solubilized from rat renal apical plasma membrane (brush-border membrane) with $C_{12}E_8$ and reconstituted into liposomes by removing the detergent from mixed micelles by hydrophobic chromatography on Amberlite XAD-4. The reconstitution was optimised with respect to the protein concentration, the detergent/phospholipid ratio and the number of passages through a single Amberlite column. The reconstituted carnitine transporter catalysed a first-order antiport reaction (carnitine/carnitine or carnitine/substrate) stimulated by external, not internal, Na^+, with a positive cooperativity. Na^+ was co-transported with carnitine. Optimal activity was found between pH 5.5 and pH 6.0. The sulfhydryl reagents MTSES, MTSET and mercurials strongly inhibited the transport. Substrate analogues inhibited the transport; the most effective were acylcarnitines and betaine, followed by dimethylglicine, tetraethylammonium and arginine. Besides carnitine, only acylcarnitines and betaine were efficiently translocated. The Km for carnitine on the external and internal side of the transporter was 0.08 and 1.2 mM, respectively. The transporter is asymmetrical and it is unidirectionally inserted into the proteoliposomal membrane with an orientation corresponding to that of the native membrane. The reconstituted carnitine transporter corresponds, very probably, to the OCTN2 protein.
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Keywords: Plasma membrane; Transport; Liposome; Reconstitution; Carnitine; OCTN2

1. Introduction

Carnitine transport across the plasma membrane of mammalian cells is an essential requisite for the maintenance of the carnitine homeostasis that results from the balance among endogenous synthesis, absorption from dietary sources, elimination and reabsorption by the kidney. The homeostasis allows carnitine to fulfil important metabolic functions, the most known of which are: (i) β-oxidation of fatty acids in mitochondria and peroxisomes, (ii) elimination of selective acyl residues, (iii) modulation of CoASH/acylCoA ratios [1–3]. Carnitine transport systems for intestinal absorption and renal reabsorption are widely distributed in mammalian tissues. After preliminary studies on carnitine uptake in liver and heart cells or in brush-border membrane vesicles ([4,5]; see Refs. [1,6] for reviews), three members of the OCTN sub-family, which encode carnitine transport proteins, have been recently cloned ([7–9]; see Ref. [10] for a review).

The OCTN's tissue distribution has been investigated. RT-PCR, Western blot analysis and in situ hybridization studies, in mouse or rat, showed that OCTN1 and -2 are expressed in kidney, whereas OCTN3 is strongly expressed in testis and not in kidney [9–11]. Furthermore, by immunohistochemical method, OCTN2 has been localized on the apical (not on the basolateral) membrane of the renal epithelial cells [12,13]. Several mutations of the OCTN2 gene are known; they cause primary carnitine deficiency ([14,15]; see Refs. [2,10] for reviews).

Functional studies of plasma membrane carnitine transporters have been performed till now in brush-border vesicles or in (over)expressing cell systems [8,9,11–13,16–19]. By these experimental approaches, some properties of the OCTN2 protein have been clarified: for example, it catalyses a sodium-dependent carnitine

Abbreviations: *p*-OHMB, *p*-hydroxymercuribenzoate; *p*-CMBS, *p*-chloromercuribenzenesulfonate; MTSES, sodium(2-sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; C_xE_y, alkyl(*x*)-poly(*y*)oxyethylene ether

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transport; substrates like acylcarnitines, betaine, choline and tetraethylammonium inhibit the carnitine transport. However, there is still contradictory information about the transport of carnitine and its derivatives across the cell plasma membrane [2,3,10]. So far, the procedure of reconstitution into liposomes has never been applied to these transporters; indeed, it has been successfully used for exhaustive functional characterization of several mitochondrial transport systems, both extracted from native membranes [20,21] or deriving from bacterial overexpression [22–26], including the mitochondrial carnitine/acylcarnitine carrier [27,28] that is essential for the β-oxidation of fatty acids. The reconstitution into liposomes has also been used for the functional study of some plasma membrane transport systems, other than carnitine transporters [29–36]. With the aim to provide an additional tool for the study of functional properties of the plasma membrane carnitine transporter(s), we have pointed out a procedure for efficient and reproducible reconstitution into liposomes of a carnitine transport system, extracted by detergent solubilization from the apical membrane of tubular renal cells (brush-border membranes). Novel functional aspects of this transporter, most probably corresponding to OCTN2, are revealed; furthermore, the procedure described represents a tool for functional studies of carnitine transporters not only from native membrane, but also from overexpressing cells.

2. Materials and methods

2.1. Materials

Amberlite XAD-4, egg yolk phospholipids (3-*sn*-phosphatidylcoline from egg yolk) and $C_{12}E_8$ were purchased from Fluka; L-[^{3}H] carnitine from Amersham; cardiolipin, Sephadex G-75, Triton X-100, Triton X-114, L-carnitine and its acyl derivatives from Sigma. All other reagents were of analytical grade.

2.2. Solubilization of the carnitine transporter

Brush border membranes were prepared from rat kidney as previously described [37]. The enrichment of apical membrane was assessed by the alkaline phosphatase specific activity; in the preparation used for the experiments here described, an increase of 15–18-fold of the specific activity of the alkaline phosphatase, with respect to the homogenate, was measured. Contamination of the brush border membrane by mitochondria was excluded due to the absence of glutamate dehydrogenase activity. Membrane preparations were divided into aliquots of 50 μl and stored at −80 °C. The carnitine transporter was solubilized by treating the membrane preparation with 2% $C_{12}E_8$ in a buffer containing 10 mM HEPES/Tris pH 7.0 and centrifuged at 13 000×*g* for 15 min at 4 °C. The supernatant (extract) was used for the reconstitution.

2.3. Reconstitution of the carnitine transporter into liposomes

The carnitine transporter was reconstituted by removing the detergent with a hydrophobic chromatography column [20,21]. In this procedure the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-4 column. The composition of the initial mixture used for reconstitution was: 25 μl of the solubilized protein (15–25 μg protein in 2% $C_{12}E_8$), 85 μl of 10% $C_{12}E_8$, 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as described in Ref. [20], 10 mM L-carnitine, 20 mM HEPES/Tris (pH 6.0) in a final volume of 700 μl. After vortexing, this mixture was passed 15 times through the same Amberlite column (0.5×3 cm) preequilibrated with a buffer containing 20 mM HEPES/Tris (pH 6.0) and 10 mM L-carnitine. All the operations were performed at 4 °C, except the passages through Amberlite, which were carried out at room temperature.

2.4. Transport measurements

To remove the external substrate, 550 μl of proteoliposomes was passed through a Sephadex G-75 column (0.7×15 cm) preequilibrated with 10 mM HEPES/Tris (pH 6.0). Transport was started by adding [^{3}H] carnitine at the indicated concentrations to the proteoliposomes and stopped by adding 50 μM mersalyl at the desired time interval. In control samples the inhibitor was added at time zero according to the inhibitor stop method [38]. The assay temperature was 25 °C. Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-75 column (0.6×8 cm) in order to separate the external from the internal radioactivity. Liposomes were eluted with 1 ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted. The experimental values were corrected by subtracting the respective control. The mersalyl-insensitive radioactivity associated to the control samples was always less than 15% with respect to the mersalyl-sensitive carnitine transport. For kinetic determinations, the initial rate of transport was measured by stopping the reaction after 10 and 20 min, i.e., within the initial linear range of [^{3}H]carnitine uptake into the proteoliposomes.

2.5. Other methods

The protein concentration was determined by the modified Lowry procedure [39]. The internal volume of the proteoliposomes was measured as previously described [20,40].

3. Results

3.1. Optimal conditions for reconstitution

To solubilize the carnitine transporter in active form, several non-ionic detergents were tested: Triton X-100, Triton X-114, $C_{12}E_8$, C_8E_4, lauryl (dimethyl) amine *N*-oxide, *n*-octyl polydisperse oligo-oxyethylene. The most suitable for the solubilization of the protein in active form was $C_{12}E_8$ at a concentration of 2%. Triton X-100 led to the extraction of active protein with 70% activity with respect to $C_{12}E_8$; Triton X-114 led to the extraction of apparently active protein: however, in this case, a higher level of mersalyl-insensitive radioactivity associated to the control samples (see Materials and methods) was measured, probably due to a higher permeability of the proteoliposomes formed in the presence of this detergent; the other detergents were nearly ineffective in the solubilization of active carnitine transporter (experiments not shown).

The reconstitution procedure has been optimised for the carnitine transporter by adjusting the parameters that influence the efficiency of transport protein incorporation into the liposomes [20,21]. In these experiments, both the transport at 15 min and the total transport after 120 min were measured. The first parameter, which is proportional to the transport rate (see Materials and methods and Fig. 2), gives information on the specific activity of the transporter, whereas the second is correlated to the number (and/or size) of the liposomes loaded with active carrier. Transport was, in any case, measured as [^{3}H] carnitine/carnitine (10 μM/10 mM) antiport. The total intraliposomal volume has also been measured to verify the efficiency of reconstitution.

In Fig. 1 the influence of the protein concentration on the reconstituted transporter is shown. Both the transport rate and the total transport increased with the protein concentration up to about 25 μg/ml. Above this value, a plateau and/or a little reduction of both the transport rate and the total transport was observed. The intraliposomal volume decreased by increasing the protein concentration, indicating that higher concentrations of protein cause the formation of less or smaller liposomes; thus, for the experiments, we have chosen a protein concentration of about 20 μg/ml, which represents a good compromise between optimal activity and internal volume.

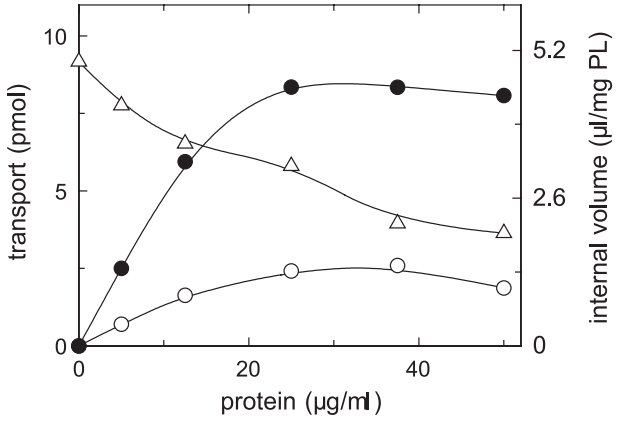

Fig. 1. Dependence of the efficiency of reconstitution of the carnitine transporter on protein concentration. The reconstitution was performed as described in Materials and methods except that increasing concentrations of protein were used. Transport was measured as 10 μM [^{3}H]carnitine uptake into proteoliposomes containing 10 mM carnitine, in the presence of 50 mM external NaCl, in 15 min (○) or in 120 min (●); (△) internal volume of the proteoliposomes. Similar results were obtained in three different experiments.

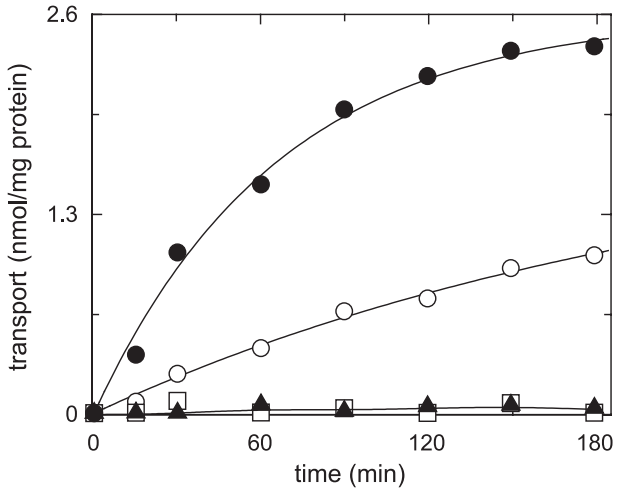

Fig. 2. Time course of [^{3}H]carnitine uptake by reconstituted proteoliposomes. 10 μM [^{3}H]carnitine was added at time zero to proteoliposomes containing 10 mM internal carnitine (○,●) or without internal substrate (□, ▲), in the presence (●, ▲) or in the absence (○, □) of 50 mM external NaCl; the transport reaction was stopped at the indicated times, as described in Materials and methods. Similar results were obtained in three different experiments.

Other critical parameters for the method of reconstitution used in this work are the detergent/lipid ratio and the number of passages of the reconstitution mixture through the Amberlite column. Broad optimal conditions were found at detergent/lipid ratio of 0.9 and 15 column passages (experiments not shown).

Cardiolipin has been reported to be essential for the activity of the mitochondrial carnitine carrier [28,41,42]. This phospholipid did not exert any influence on the activity of the reconstituted carnitine transporter from plasma membrane upon addition during the solubilization or the reconstitution at concentrations ranging from 20 to 200 μg/ml (not shown).

3.2. Functional characterization

The dependence of the transport activity of the carnitine transporter has been studied as function of the time of incubation of the proteoliposomes with 10 μM external labelled carnitine under different experimental conditions: in the presence or in the absence of intraliposomal carnitine and in the presence or in the absence of external NaCl. The results are summarized in Fig. 2. The accumulation of [^{3}H]

carnitine into the proteoliposomes containing internal carnitine increased as a function of time; on the contrary, virtually no accumulation of radioactivity was observed in the vesicles without internal substrate, indicating that the transport process occurs by an antiport mode (carnitine/carnitine homoexchange). The [^{3}H] carnitine/carnitine antiport was strongly stimulated by the presence of external NaCl; in this case the radioisotopic equilibrium was reached after about 160 min; the experimental data describing the transport of carnitine in the absence and in the presence of external NaCl, both fitted a first order rate equation; the first order rate constant, k, resulted to be 0.005 and 0.013 min^{-1}, the transport rate (calculated as the product of k and the transport at equilibrium) was 8.5 or 36.4 pmol/min/mg protein in the absence or in the presence of NaCl, respectively.

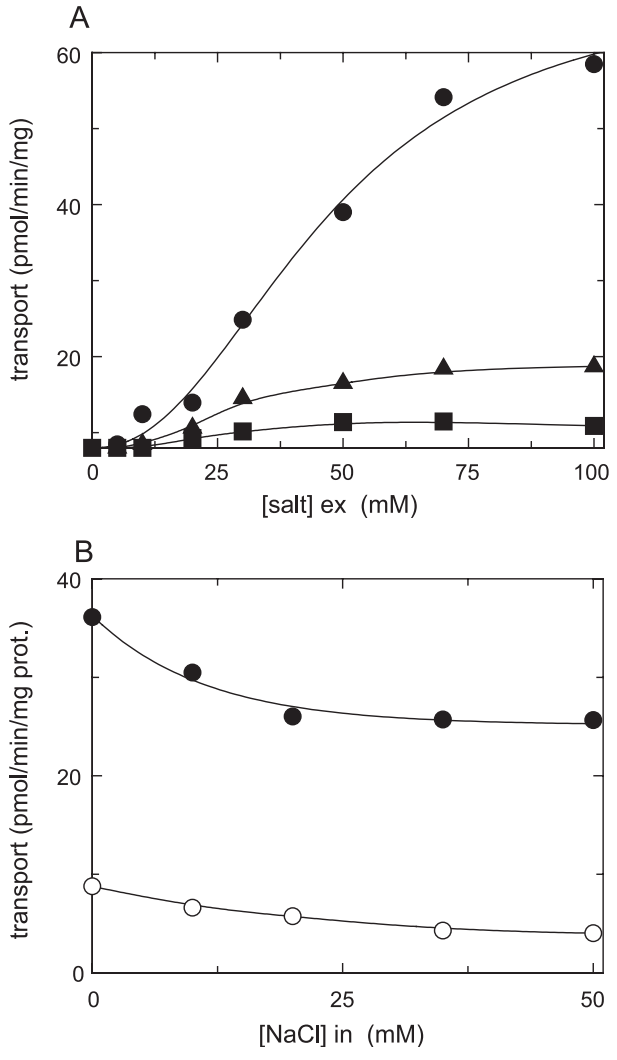

Fig. 3. Dependence of carnitine transport on the concentration of NaCl in the extraliposomal (A) or intraliposomal (B) compartment. Transport rate was measured as 10 μM [^{3}H]carnitine uptake into proteoliposomes containing 10 mM carnitine, as described in Materials and methods. In (A), NaCl (●), LiCl (▲) or KCl (■) was added at the indicated concentrations outside the proteoliposomes; in (B), NaCl was included at the indicated concentrations inside the proteoliposomes in the reconstitution mixture and the transport rate was measured in the absence (○) or in the presence (●) of external 50 mM NaCl. Similar results were obtained in four different experiments.

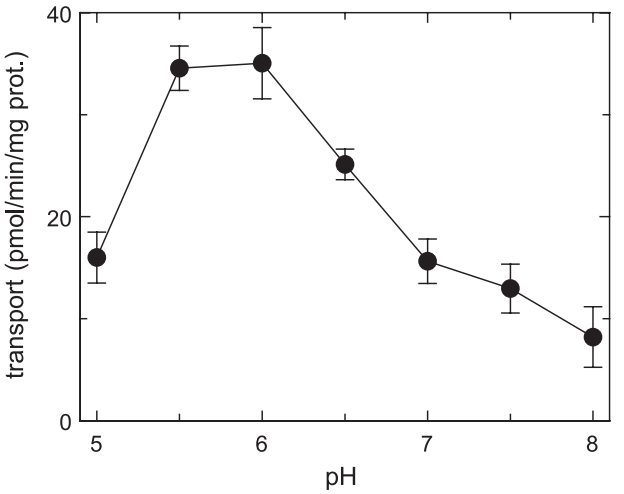

Fig. 4. Effect of pH on the reconstituted carnitine transporter. All the experimental procedures from the reconstitution to the transport measurement (see Materials and methods) were performed in 20 mM HEPES/Tris buffer at the indicated pH. Transport rate was measured as 10 μM [^{3}H] carnitine uptake into proteoliposomes containing 10 mM carnitine, in the presence of 50 mM external NaCl. The results are means±S.D. of triplicate samples of one experiment. Similar results were obtained in three different experiments.

To gain further insight on the stimulation of the carnitine antiport, the dependence of the transport on the concentration of externally added cations, as Cl^- salts, was studied. The data of Fig. 3A show that the transport rate of carnitine antiport, starting from about 8 pmol/min/mg protein at 0 mM salt, was strongly stimulated by external NaCl, reaching a value of about 60 pmol/min/mg protein at 100 mM NaCl. The stimulation followed a sigmoidal dependence on the NaCl concentration. The data were interpolated by the Hill allosteric kinetic equation; half maximal effect was given by 48 mM NaCl (52±8.5 from 4 experiments) and the cooperativity index was 2.1 (1.8±0.4 from 4 experiments). LiCl exerted a stimulatory effect much lower than that of NaCl; KCl and (not shown) NH_4Cl, tetraethylammonium (chloride) and choline (chloride), had a negligible effect. In other experiments it was found that the stimulation of carnitine transport, by 25 mM Na_2SO_4 or 50 mM NaAcetate, was coincident to that of 50 mM NaCl (not shown) demonstrating, together with the data of Fig. 3A, that the stimulatory effect is due to the Na^+ cation. In a different experiment we studied the dependence of the transport on internal NaCl concentration, both in the presence and in the absence of external Na^+. As shown in Fig. 3B, Na^+ did not stimulate the transport when present inside the proteoliposomes, nor in the presence neither in the absence of external Na^+. If any, a light inhibition effect was observed at higher internal Na^+ concentrations. Similar results were obtained with Na_2SO_4 or NaAcetate (not shown).

To verify whether or not Na^+ were co-transported with carnitine, external [^{3}H] carnitine, together with [^{22}Na]Cl, was incubated with the proteoliposomes. At Na^+ concentrations ranging from 1 to 10 mM, the time courses of carnitine and Na^+ were nearly coincident, indicating a 1:1 stoichiometry of transport (experiment not shown).

The dependence of the carnitine antiport on the pH has been studied (Fig. 4). Maximal transport activity was observed in an acidic pH range between pH 5.5 and pH 6.0. Out of this range, the transport activity was much lower.

The sensitivity of the carnitine antiporter to inhibitors was studied. Inhibitors were chosen among protein-modifying reagents or molecules structurally related with the substrate. As reported in Table 1, the carnitine antiport was strongly inhibited by known SH reagents; the mercurials $HgCl_2$, mersalyl, *p*-OHMB and *p*-CMBS and the methanthiosulfonates MTSES and MTSET were the most effective; other SH reagents like *N*-ethylmaleimide and *N*-phenylmaleimide had a lower inhibitory effect, even though by increasing their concentration up to 0.5 mM an inhibition of 86% and 74% was observed with *N*-ethylmaleimide and *N*-phenylmaleimide, respectively. Pyridoxal-5-phosphate, which reacts with Lys amino groups, and bathophenanthroline, a metal chelating reagent, exerted a low inhibitory effect even at very high concentrations. Among the substrate analogues, carnitine acyl esters were tested; acetylcarnitine led to about 50% inhibition at 0.1 mM, i.e. at a concentration close to the Km of the transporter for carnitine (see below); 5 mM acetylcarnitine led to complete inhibition; 5 mM propionylcarnitine, octanoylcarnitine and decanoylcarnitine led to a nearly com-

Table 1
Effect of inhibitors on the reconstituted carnitine transport

Inhibitor	Concentration (mM)	Inhibition (%)
$HgCl_2$	0.05	88±7.8
Mersalyl	0.05	100
p-Hydroxymercuribenzoate	0.05	93±10
p-Chloromercuribenzensulfonate	0.05	98±13
MTSET	0.05	101±9.1
MTSES	0.05	99±12
N-Ethylmaleimide	0.05	38±3.5[a]
N-Ethylmaleimide	0.5	86±11
N-Phenylmaleimide	0.05	21±4.2[a]
N-Phenylmaleimide	0.5	74±8.6[a]
Bathophenanthroline	10	54±8.4[a]
Pyridoxal-5-phosphate	15	27±6.7[a]
L-Acetylcarnitine	0.1	51±7.5[a]
L-Acetylcarnitine	1	91±9.7
L-Acetylcarnitine	5	101±8.3
L-Propionylcarnitine	5	95±10
L-Octanoylcarnitine	5	96±7.4
L-Decanoylcarnitine	5	92±8.6
L-Palmitoylcarnitine	0.1	37±12[a]
L-Palmitoylcarnitine	1	82±12
Betaine	1	92±9.0
Dimethylglycine	1	48±6.3[a]
Glycine	1	22±4.1[a]
Tetraethylammonium	1	43±3.5[a]
Choline	1	27±3.3[a]
Ammonium chloride	1	23±8.8[a]
Arginine	1	42±5.4[a]
Creatine	1	17±4.0[a]
Trimethyl-lysine	1	14±3.5[a]

Transport was measured as 10 μM [^{3}H] carnitine uptake into proteoliposomes, reconstituted as described in Materials and methods, containing 10 mM internal carnitine, in the presence of 50 mM external NaCl, in 40 min. The inhibitors were added 1 min before the labelled substrate at the concentrations indicated. Percent of transport inhibition was calculated for each experiment with respect to the control sample (referred as 100%), i.e. proteoliposomes treated with 0.05 mM mersalyl. The results are means±S.D. of the percentages for three experiments. The average transport activity of the control samples of the three experiments analysed was 1200±422 pmol/40 min/mg protein.

[a] Significantly different from the control (100%) as estimated by Student's *t* test ($P<0.05$).

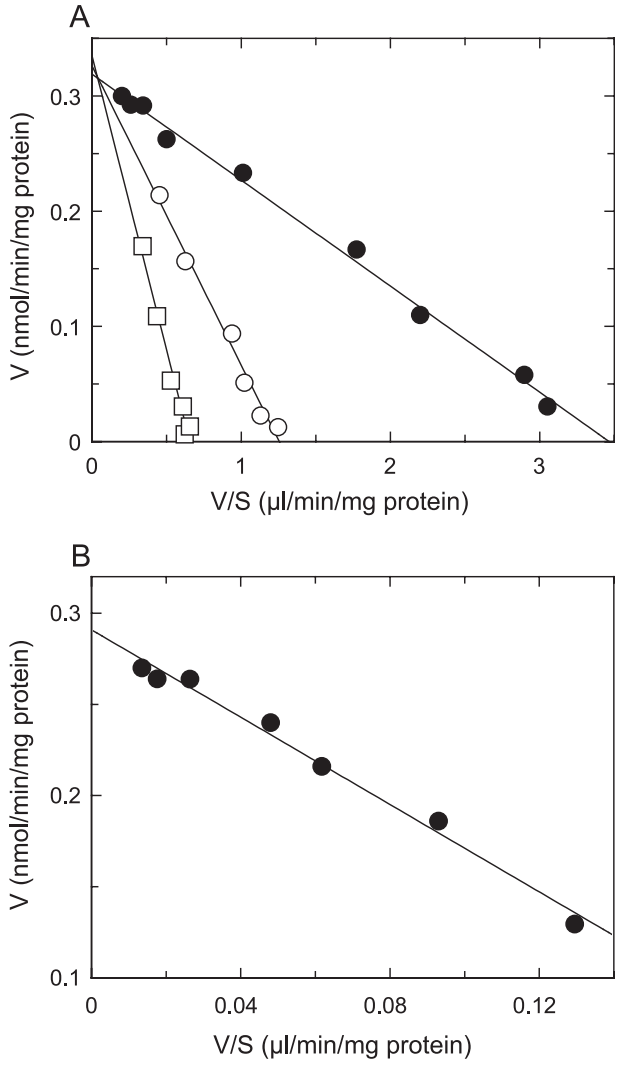

Fig. 5. Dependence on substrate concentration of the rate of carnitine antiport. [^{3}H]carnitine at the indicated concentration was added to proteoliposomes containing 10 mM internal carnitine in the absence (●) or in the presence of 0.1 mM acetylcarnitine (○), 0.3 mM betaine (□), (A); or 0.5 mM [^{3}H]carnitine was added to proteoliposomes containing internal carnitine at the indicated concentrations (B); the transport rate was measured, as described in Materials and methods, in the presence of 50 mM external NaCl. Data were plotted according to Eadie-Hofstee. Similar results were obtained in five different experiments.

plete inhibition; palmitoylcarnitine was used at 0.1 and 1 mM since it has a detergent effect at higher concentrations [28]; at the concentration tested, it was little less effective than acetylcarnitine, i.e., the affinity of the carrier towards long-chain acylcarnitines was slightly lower than that for carnitine and acetylcarnitine. Betaine also was a very effective inhibitor; the removal of *N*-methyl groups led to a gradual decrease of inhibition in dimethylglycine and glycine. A reduction of inhibition was also observed in the absence of the carboxyl group; tetraethylammonium had a middle effect, choline and ammonium chloride were poor inhibitors. The amino acid arginine had an effect similar to tetraethylammonium; creatine and trimethyl-lysine had a very poor, if any, effect. No significant protection of mersalyl or *N*-ethylmaleimide inhibition by carnitine was found, indicating that the SH residues should not be located in the active site. By studying the carnitine transport kinetics in the absence and presence of acetylcarnitine or betaine (see Fig. 5A), competitive inhibition was found, indicating that these inhibitors interact with the active site.

To establish which of the inhibitors could also be transported, the experiment of Table 2 has been performed. In this experiment, the ability of the internal substrates to be exchanged with external labelled carnitine has been tested. To compare the effect of palmitoylcarnitine (that cannot be used at concentrations higher than 1 mM) with that of the control, two conditions of internal carnitine concentration (controls) were used: 1 and 10 mM. As clearly demonstrated by the data in Table 2, the carnitine acyl derivatives and betaine were efficiently transported in antiport for external carnitine; on the contrary, with the exception of tetraethylammonium that was transported with a very low efficiency, all the other substrates tested were not transported, in spite of the inhibitory effect observed in the experiment of Table 1.

Table 2
Dependence on internal substrate of carnitine transport in reconstituted liposomes

Internal substrate	Concentration (mM)	Carnitine transport (% of the control)
None		<5
L-Carnitine	1 or 10	100
L-Acetylcarnitine	1	98±8.7
L-Acetylcarnitine	10	102±9.9
L-Propionylcarnitine	10	96±11
L-Palmitoylcarnitine	1	93±13
Dimethylglycine	10	<5
Glycine	10	<5
Betaine	10	81±7.5[a]
Choline	10	<5
Creatine	10	<5
Arginine	10	<5
Tetraethylammonium	10	16±4.2[a]
Ammonium chloride	10	<5
Trimethyl-lysine	10	<5

Internal substrates were included in the reconstitution mixture (see Materials and methods). After the removal of external substrate on Sephadex G-75, transport was measured as 10 μM [^{3}H] carnitine uptake into the proteoliposomes in the presence of 50 mM external NaCl, in 40 min. Percent of carnitine transport was calculated for each experiment with respect to its control sample (referred as 100%), i.e. proteoliposomes containing 1 mM or 10 mM internal carnitine. The results are means±S.D. of the percentages for three experiments. The average transport activity of the control samples of the three experiments analysed was 259±117 pmol/40 min/mg protein at 1 mM internal carnitine or 1390±568 pmol/40 min/mg protein at 10 mM internal carnitine.

[a] Significantly different from the control (100%) as estimated by Student's *t* test ($P<0.05$).

To obtain the basic kinetic data of the carnitine transporter, the dependence of the antiport rate on both external and internal substrate concentration was studied by changing alternatively the carnitine concentration in one compartment at fixed (close to saturation) carnitine concentration on the opposite compartment. The experimental data were plotted according to Eadie-Hofstee; in this representation the Km value corresponds to the slope and, hence, the hypothetical presence of more than one Km is easily detectable. As shown by Fig. 5A and B, on both sides of the membrane, only one slope (Km) is measured. The Km outside is much lower than the Km inside: the mean value from five different experiments was 80±11 μM or 1.2±0.25 mM on the external or the internal side, respectively. The Vmax values from the two experiments were similar. The mean value from 10 different experiments was 350±162 pmol/min/mg protein. The standard deviation of the Vmax was very high when comparing different experiments, probably due to variations in the amount of active transport protein molecules present in each preparation.

4. Discussion

In this paper we have studied the carnitine transporter from renal cell plasma membrane by reconstitution into liposomes. The reconstitution system leads to some advantages, with respect to intact cells or brush-border membrane vesicles, like the possibility to control the experimental conditions in the internal compartment, which allows the determination of both the internal substrate affinity or the effect of various molecules; the reduction of interferences due to the virtual absence of external and internal enzymes; a longer radioactivity equilibration time (especially with respect to brush-border membrane vesicles), due to the low protein/lipid ratio (one or few protein per liposome), which leads to a better kinetic resolution; the possibility of modifying the lipid composition of the membrane; the potential application on expressed transporters. In addition, the reconstitution procedure here used leads to the formation of proteoliposomes larger than those obtained by other methods like the freeze-thaw sonication [20,21]; this feature allows to increase the accumulation of radioactivity inside the active vesicles, i.e. the proteoliposomes containing active transporters inserted into the membrane.

We have identified the reconstituted carnitine transporter as the rat OCTN2 for the following reasons: (i) the source of

the reconstituted carnitine transporter (see Materials and methods) corresponds to the location of the OCTN2 [9,11–13]; (ii) the Na^+ stimulation and the specificity for carnitine, its acyl derivatives, betaine, tetraethylammonium and choline are nearly coincident to those reported for the rat OCTN2 expressed in cells [11] and very similar to those of OCTN2 from other sources [8,9,16,19]; (iii) the ratio between the transport of carnitine and that of the poor substrate tetraethylammonium (6.25) is closer to the ratio reported for mouse OCTN2 than to those of mouse OCTN1 and OCTN3 [9,19]. On the other hand, different functional features are reported for OCTN1 [10] and different features and location for OCTN3 [9]. In addition, we can exclude that the carnitine transporter is an unknown member of the OCTN sub-family, since no further member, besides OCTN1-3, can be identified up to date, by aligning the rat OCTN2 cDNA or protein with all non-redundant data bases (http://www.ncbi.nlm.nih.gov/BLAST/); see also [9].

By using the optimised reconstituted system, we have provided additional functional information on the transport system or have clarified some uncertainties previously reported [2,3,10,43]. Interestingly, we have found that it catalyses a peculiar mode of transport: an obligatory antiport of carnitine and other substrates (Fig. 4 and Tables 1 and 2), coupled to external (not internal) Na^+ co-transport with carnitine; this mechanism is in agreement with previous conclusions on the OCTN2 overexpressed in cells [19]. The reconstituted carnitine transporter has a very low tolerance for Li^+ as found for OCTN2 in cells [8,13]. For optimal translocation efficiency, the substrates must fulfil at least two requirements: the presence of a carboxylic group (the absence of this functional group in the choline does not allow transport); the presence, on the opposite end of the molecule, of a charged three-substituted amino group (dimethylglycine that lack only one *N*-methyl group interacts and inhibits the transporter, but is not translocated). Differently, the distance between the two essential groups is not very critical: one (betaine) or three (carnitine) carbon atoms between the carboxylic and the amino group are accepted; the presence of an acyl group esterified with the β-hydroxyl group of carnitine is accepted without apparent restrictions. The substrate specificity of the carnitine plasma membrane transporter towards acylcarnitines is quite different from that of the mitochondrial carnitine/acylcarnitine carrier; the first shows highest affinity for carnitine and short-chain acylcarnitines; on the contrary, the second has an affinity towards long-chain acylcarnitines much higher than that for carnitine and short-chain acylcarnitines [27]. Furthermore, the carnitine transporter efficiently catalyses the transport of betaine, the mitochondrial carrier does not (Indiveri, C. et al. unpublished results). The pH dependence of the reconstituted carnitine transporter is different from that reported for the mouse OCTN2 in cell system [9]; however, as stated by the author themselves, the pH dependence of OCTN2 in cells was in contrast with the acidic pH of the renal tubules; on the contrary, the pH dependence found here is in good agreement with the physiological context.

The external Km value found in the reconstituted system is similar, or higher than, values reported for the carnitine transporter in brush-border vesicles ([10] and see Ref. [17] for references), similar to a value reported for OCTN2 expressed in RBE4 cells [18] and higher than the values reported for OCTN2 from various sources expressed in different cell systems [8,11–13]. The first determination of internal Km is described here: it is about one order of magnitude higher than the external Km; thus, the transporter is functionally asymmetrical in agreement with the sidedness of the Na^+ regulation. It has an unidirectional orientation, since a single Km value is found on each side of the membrane and no activation by internal Na^+ can be observed. The orientation of the reconstituted transporter should correspond to that of the native membrane since, as in the cell, the Na^+ regulation site is located outside the proteoliposomes. The functional asymmetry corresponds to the structural asymmetry of the OCTN2 transport protein, deduced on the basis of the hydropathy profile analysis [2,8,10]. The protein has 12 transmembrane α-helices connected by hydrophilic loops of very different size on the two sides of the transporter. On the basis of the sequence analysis, the larger hydrophilic loop should be located on the external side of the cell, due to the presence of potential glycosylation sites [2,8]. The inhibition of the reconstituted transporter, caused by externally added membrane impermeant [44] SH reagents MTSES, MTSET and *p*-CMBS (Table 1), can only be explained by the exposure of Cys residues towards the extraliposomal side. Since all the four Cys in the hydrophilic moiety of the OCTN2 protein (the remaining 2 Cys are in the transmembrane segments) are located in the large extracellular loop that carries the glycosylation sites, the extracellular loop must also be extraliposomal.

The data described are in agreement with a physiological role of this transporter in the reabsorption of carnitine from the tubular lumen by exchanging it with molecules like carnitine acyl esters or other metabolites, which must be excreted [1–3,6]. Thus, the export of these metabolites does not occur by passive diffusion as previously hypothesized (see Refs. [2,3] for reviews). The transport mechanism of the carnitine transporter, i.e., the antiport coupled with Na^+ co-transport, allows to fulfil the function by a single transporter and to reduce the osmotic imbalance that may be generated by the simple co-transport of carnitine plus Na^+.

This paper, in our knowledge, represents the first example of reconstitution of a carnitine transporter from plasma membrane.

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