Biochimica et Biophysica Acta, 471 (1977) 296—304
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BBA 77858

SPECIFICITY AND CHARACTERISTICS OF THE CARNITINE TRANSPORT IN HUMAN HEART CELLS (CCL 27) IN CULTURE

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(Received May 16th, 1977)

Summary

The specificity and characteristics of the uptake mechanism for radiolabeled L-carnitine has been studied in cultured human heart cells (CCL 27). Iodoacetate, 2,4-dinitrophenol, arseneoxide and potassium cyanide do not reduce the uptake significantly, while sodium fluoride in high concentration (25—50 mM) inhibits the transport. Sulfhydryl blocking agents like *N*-ethylmaleimide, 2,4-dinitrofluorobenzene and Ellman reagent [5,5-dithiobis-(2-nitrobenzoic acid)] all reduce the uptake of radiolabeled carnitine; while phloridzin (0.1 mM), ouabain or amino acids (1—5 mM) do not. Thus it seems that the carnitine transport depends upon free sulfhydryl groups, and is neither linked to the transport of amino acids or glucose, nor to the activity of $(Na^+ + K^+)$-ATPase. Variation in osmolality in the incubation medium within 225 to 450 mosM/kg water does not influence the uptake. An increase in pH from 7 to 8 reduces the transport approx. 40%. Compounds structurally related to carnitine, containing a trimethylamino group and a carboxylic group, reduce the uptake. L-Carnitine has a greater affinity for binding to the transport mechanism than D-carnitine. Acylcarnitines with varying acyl group length inhibit the transport, and the L isomers more than their D counterparts. The 50% inhibiting concentration on the uptake of 2 μM L-carnitine, was 90 μM for betaine, $11 \cdot 10^3$ μM for choline, 2 μM for butyrobetaine, 20 μM for D-carnitine, 14 μM for 5-trimethylaminovaleric acid, 8 μM for L-acetylcarnitine and 3 μM for L-palmitoylcarnitine. Radiolabeled L-acetylcarnitine is also transported into the cells with a K_m of 8 μM and a V of 10 pmol $\cdot$ μg^{-1} DNA $\cdot$ h^{-1}. There is a competitive type of inhibition between L-carnitine and L-acetylcarnitine. Since L-acetylcarnitine constitutes 30% of total carnitine in rat plasma, both L-carnitine and L-acetylcarnitine can be physiological substrates for this active transport mechanism in vivo.

Introduction

Carnitine is synthesized in the liver and rapidly transported by the bloodstream to different tissues [1]. Different organs vary greatly in their intracellular concentration of carnitine. The heart has a high concentration of carnitine, 60-fold that in plasma [1—3]. However, in some diseases the concentration of carnitine in the heart is reduced [5—7]. The concentrative mechanism described, especially active in human heart cells, might explain the high concentration of carnitine found in the heart in vivo [4].

These studies have been undertaken to investigate further this concentrative mechanism.

Materials and Methods

Materials

L-[*Me*-^{3}H]Carnitine (spec. act. 100 Ci/mol), L-[*Me*-^{3}H] acetylcarnitine (spec. act. 100 Ci/mol), methylcholine, DL-norcarnitine, dimethylamino-2-propanol and dimethylaminobutyric acid were gifts from Prof. J. Bremer, University of Oslo, Norway. Other compounds structurally related to carnitine were gifts from Prof. G. Lindstedt, Dept. of Clinical Chemistry, University of Gotenburg, Sweden. ϵ-*N*-Trimethyl-lysine was a gift from Dr. H.T. Haigler, Dept. of Biochemistry, Vanderbilt University, Nashville, Tenn., U.S.A. L- and D-carnitine were gifts from Otsuka Pharmaceutical Company, Osaka, Japan. Butyrobetaine and Ellman reagent [5,5-dithiobis-(2-nitrobenzoic acid)] were obtained from Sigma Chemicals Co., St. Louis, U.S.A. D- and L-acylcarnitines were synthesized as earlier described [8]. Other reagents were commercially available products of analytical grade. The Girardi human heart cells (CCL 27) were obtained from American Type Culture Collection Cell Repository, Rockville, Md.,U.S.A.

Methods

The heart cells were cultured and the L-[^{3}H]carnitine uptake was determined as previously described [4], with the following modifications. The cells were transferred to Costar Tissue Flasks one day prior to the experiments. Before the experiments, the cells were washed twice with 3 ml medium devoid of serum to remove carnitine present in the serum. The monolayer was removed from the flasks by using 4 ml of a solution of 0.02% trypsin in Versene buffer (NaCl 140 mM, KH_2PO_4 3 mM, KCl 5 mM, Na_2HPO_4 16 mM, EDTA 1 mM) for 5 min at 37°C. The cell pellet was washed twice with medium devoid of serum to remove extracellular carnitine, then heated to 100°C for 30 min in 10% trichloroacetic acid for complete extraction of intracellular carnitine and DNA. The precipitate was removed by filtration through a blueband filter. The DNA content was determined according to Burton [9], and the amount of radiolabeled carnitine taken up was related to μg DNA in the cells.

Carnitine and acylcarnitines were separated as described by Christiansen and Bremer [10]. Cellular protein was determined by the method of Lowry et al. [11].

Radioactivity was determined by dissolving the samples in Diluene scintillator solution (Packard Instrument Co. Ill.) and counted in a Packard Tri-Carb

scintillation spectrometer, corrected for quenching by the channel ratio method.

Results and Discussion

The effect of osmolality and pH

High osmolality (above 450 mosM/kg water) or low osmolality (beneath 225 mosM/kg water) significantly reduced the uptake of L-carnitine (Fig. 1). An increase in pH from 7 to 8 resulted in a decrease in the uptake of L-carnitine of approx. 40% (Fig. 2). No attempt was made to test a wider pH range since all the incubations were done within these limits, and the variation between different samples in the same experiment was less than 0.3 pH units.

The effect of metabolic inhibitors

In previous studies metabolic inhibitors reduced the uptake of L-carnitine only moderately [4]. These studies, therefore, have been repeated and expanded.

High concentrations of iodoacetate, 2,4-dinitrophenol and arseneoxide reduced the uptake moderately, and no effect of potassium cyanide was observed (Table I). High concentrations of sodium fluoride (in accordance with previous observations [4]) reduced the uptake of L-carnitine. The lack of any appreciable effect of these inhibitors is not unexpected. The concentration gradient of carnitine across the cell membrane is approx. 300 μM [4], and the transport is approx. $5 \cdot 10^{-12}$ mol $\cdot \mu g^{-1}$ DNA $\cdot h^{-1}$. The required energy per unit time is therefore probably small. Assuming a high affinity of the transport

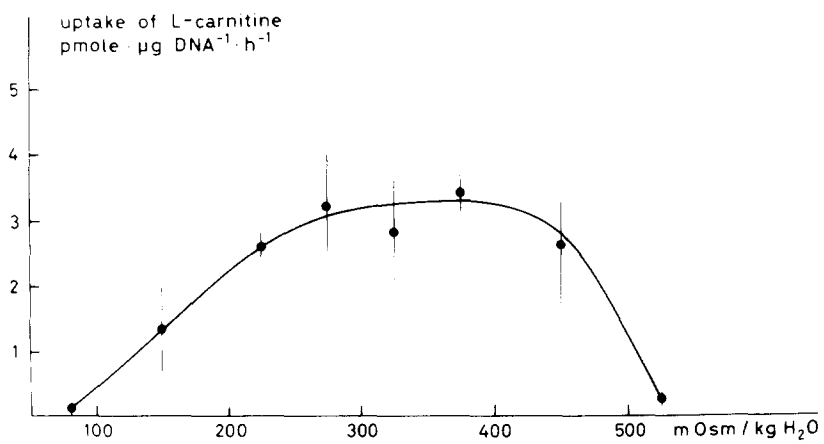

Fig. 1. The uptake of L-[^{3}H]carnitine at varying osmolality. The cells (containing 100—200 μg DNA) were incubated with [^{3}H]carnitine (2 μM) for 2 h at 37°C. Distilled water or NaCl was added to the medium to alter the osmolality, which was measured in a Knauer osmometer prior to incubation. The values represent means from four separate experiments, and vertical bars are standard deviations.

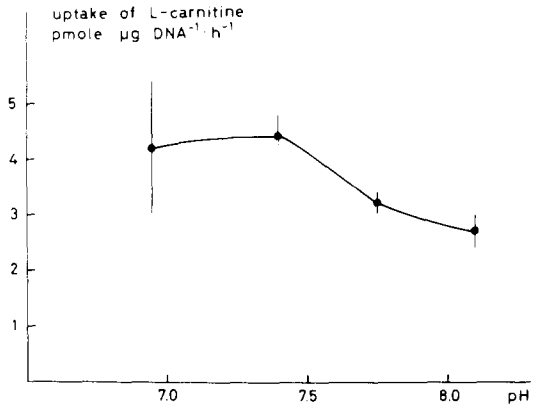

Fig. 2. The uptake of L-[^{3}H]carnitine at varying pH values. The cells (containing 100—200 μg DNA) were incubated with L-[^{3}H]carnitine (1 μM) for 30 min (to minimize pH alteration) at 37°C in Eagle's minimum essential medium with Earle's balanced salt solution with Tris · HCl (25 mM) as buffer. The pH was adjusted by varying the amount of HCl and measured before and after incubation. Each bar represents the mean of four samples with standard deviation.

mechanism for the energy yielding process, it would be difficult to deplete the cells of energy to such an extent as to make it rate limiting for the transport of carnitine. The effect of sodium fluoride in the highest concentrations (50 mM) is most likely a non-specific toxic effect.

N-Ethylmaleimide, 2,4-dinitrofluorobenzene and 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman reagent) all reduced the uptake significantly (Table I), indi-

TABLE I

THE EFFECT OF METABOLIC INHIBITORS ON THE UPTAKE OF L-[^{3}H]CARNITINE

The cells (containing 100—300 μg DNA and 300—1000 μg protein) were incubated for 2 h at 37°C with L-[^{3}H]carnitine (2 μM). The results are given as percent of the mean uptake of L-carnitine in three contols (with variation in control values in different experiments 4—8 pmol · μg^{-1} DNA · h^{-1}). The number of observations is given in parenthesis.

Inhibitor	Concn. (mM)	% Uptake of L-carnitine (mean ± SD)	
Iodoacetate	1	87 ± 10	(4)
2,4-Dinitrophenol	1	74 ± 6	(4)
As_2O_3	0.1	80 ± 8	(4)
KCN	1	124 ± 10	(4)
NaF	2.5	117, 120	(2)
	12.5	93, 94	(2)
	25	61, 64	(2)
	50	7 ± 1	(3)
N-Ethylmaleimide	0.1	38 ± 32	(6)
	1	5 ± 5	(6)
2,4-Dinitrofluorobenzene	0.5	30, 34	(2)
	5	2 ± 2	(4)
5,5-Dithiobis-(2-nitrobenzoic acid)	1	14 ± 6	(4)
Phloridzin	0.1	122 ± 17	(4)
	1	86 ± 29	(6)
	10	49, 72	(2)
Ouabain	0.1	103 ± 11	(4)

cating that free sulfhydryl groups are essential for the uptake of carnitine.

Phloridzin inhibited the transport only in high concentrations (10 mM). This is probably a non-specific toxic effect, since the cells showed evidence of toxic influence after the incubation. Ouabain did not reduce the uptake. It seems likely, therefore, that the transport of L-carnitine is neither linked to transport of glucose nor dependent on the activity of $(Na^+ + K^+)$-ATPase.

Effect of amino acids

There was no reduction in the uptake of L-carnitine by lysine, valine or aspartate added to the incubation medium. Glutamine and proline caused only a moderate inhibition (30 and 20% respectively), when added in a concentration 2500-fold that of carnitine in the medium. Thus, it is unlikely that L-carnitine transport is coupled to any of the transport mechanisms for the amino acids.

The effect of compounds structurally related to carnitine

Compounds containing a trimethylamino group and a carboxylic group, with a carbon skeleton from 2—6 carbon atoms, all reduced the uptake of L-carnitine (Table II). Similar compounds containing a dimethylamino group had no effect. (3-Dimethylaminopropionic acid, dimethylamino-2-propanol, 4-dimethylaminobutyric acid, 4-dimethylamino-1-butanol, DL-norcanitine, 5-dimethylaminovaleric acid.) Choline, which does not contain a carboxylic group, reduced the uptake of L-carnitine at much higher concentrations (Tables II and IV), while methylcholine had no effect. Butyrobetaine had, as previously described [4], an inhibitory effect (Table II). The other precursor, ϵ-*N*-trimethyllysine showed no effect even in high concentrations (1 mM).

D-Carnitine reduced the uptake of L-[^{3}H]carnitine to a moderate extent (Table III). There is an increasing inhibition by D- and L-acylcarnitines with increasing chain length of the acyl moiety. The L-acylcarnitines inhibited more extensively than their D-counterparts (Table III). L- and DL-palmitoyl-carnitine reduced the uptake of L-carnitine to 2—3% of controls. We cannot exclude, however, that some of this inhibition is due to a detergent effect on the membrane.

TABLE II

THE EFFECT OF STRUCTURALLY RELATED COMPOUNDS ON THE UPTAKE OF L-[^{3}H]CARNITINE

The cells (containing 100—200 μg DNA) were incubated for 2 h at 37°C with L-[^{3}H]carnitine (2 μM) and inhibitor (20 μM, when not otherwise stated). Uptake in controls and calculation of results as stated in Table I. Each result is based on four observations.

Compounds	% Uptake of L-carnitine (mean ± SD)
Betaine	54 ± 4
Choline (70 mM)	22 ± 2
3-Trimethylaminopropionic acid	74 ± 7
Butyrobetaine	23 ± 6
5-Trimethylaminovaleric acid	48 ± 6
6-Trimethylaminocaproic acid	57 ± 18

TABLE III

THE EFFECT OF ACYLCARNITINES ON THE UPTAKE OF L-[^{3}H]CARNITINE

The cells (containing 100—200 μg DNA) were incubated for 2 h at 37°C with L-[^{3}H]carnitine (2 μM) and acylcarnitines (20 μM). Uptake in controls and calculation of results as stated in Table I. The number of observations is given in parenthesis.

Acylcarnitines	% Uptake of L-carnitine (mean ± SD)	
D-Carnitine	53 ± 10	(4)
D-Acetylcarnitine	57 ± 10	(4)
D-Hexanoylcarnitine	30 ± 6	(4)
L-Acetylcarnitine	27 ± 5	(4)
L-Hexanoylcarnitine	21 ± 9	(4)
L-Palmitoylcarnitine	3 ± 1	(4)
DL-Palmitoylcarnitine	3, 3	(2)
L-Bromopalmitoylcarnitine	18 ± 3	(4)

Competitive studies with calculations of K_i and V for different substrates have not been undertaken due to the complexity of the system. However, a 50% inhibiting concentration has been calculated from separate experiments (Table IV). The 50% inhibiting concentrations of butyrobetaine, D-carnitine, 5-trimethylaminovaleric acid, L-acetylcarnitine and L-palmitoylcarnitine are all in the same order as the K_m for L-carnitine (5 μM) [4]. Betaine has a 50% inhibiting concentration of 10—20 times the K_m of L-carnitine, while for choline the concentration is approximately 1000 times higher. The importance of the carboxylic group for determining the affinity to the carrier, can be seen from the difference between betaine and choline.

From these experiments it seems that the carrier for the L-carnitine transport has two "recognition sites", one site which detects with high specificity the presence of a trimethylamino group, the second detects a carboxylic group within the molecule. The carrier has a preference for the L-isomers (Tables III and IV), and depends upon free sulfhydryl groups. The length of the carbon

TABLE IV

THE 50% INHIBITING CONCENTRATIONS OF DIFFERENT COMPOUNDS ON THE UPTAKE OF L-[^{3}H]CARNITINE

The cells (containing 100—200 μg DNA) were incubated for 2 h at 37°C with L-[^{3}H]carnitine (2 μM) and the inhibitor in increasing concentrations. Each determination of the 50% inhibiting concentration was based upon 4 control values (with variation from 4—8 pmol · μg^{-1} DNA · h^{-1}) of the uptake of L-[^{3}H]carnitine and three duplicate samples with increasing inhibitor concentration.

Compound	50% inhibiting concentration (μM)
Betaine	90
Choline	$11 \cdot 10^3$
Butyrobetaine	2
D-Carnitine	20
5-Trimethylaminovaleric acid	14
L-Acetylcarnitine	8
L-Palmitoylcarnitine	3

skeleton seems to be of less importance within the limits we have tested (2—6 carbon atoms).

The specificity thus found for this carrier, shares many of the aspects of specificity found for both carnitine acetyltransferase (EC 2.3.1.7) [12], carnitine palmitoyltransferase (EC 2.3.1.21) [13,14] and carnitine acylcarnitine translocase system of mitochondria [15,16]. They are all inhibited by sulfhydryl reagents and show stereospecificity. The effect of a number of compounds structurally related to carnitine are similar, though there are some differences in the requirements for methylated amino groups. The specificity found for the uptake of L-carnitine in liver cells [10], also differ from our results in this respect.

The uptake of L-[Me-^{3}H]acetylcarnitine

L-Acetylcarnitine accounts for 30% of total carnitine in rat plasma [10]. It is therefore of interest to see whether acetylcarnitine itself is transported into the cells, and not only inhibits the uptake of L-carnitine. L-[*Me*-^{3}H]Acetylcarnitine was taken up into the cells as depicted in Fig. 3. The data were fitted into a Lineweaver-Burke plot (not shown) giving a K_m of 8 μM and a V of 10 pmol · μg^{-1} DNA · h^{-1}, being of the same magnitude as the K_m and V of L-carnitine previously reported [4]. The radiolabeled L-acetylcarnitine contained approx. 7% of free L-carnitine. This impurity does not seriously affect the results, since

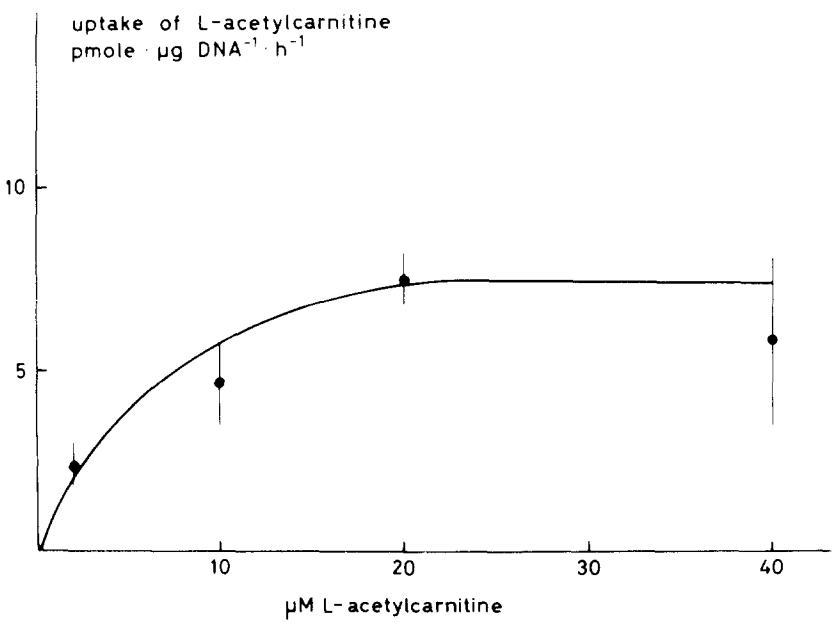

Fig. 3. The uptake of L-[^{3}H]acetylcarnitine. The cells (containing 100—200 μg DNA) were incubated for 2 h with increasing concentrations of L-acetylcarnitine. The values are means of four samples and vertical bars are standard deviations. The uptake data were fitted into a Lineweaver-Burke plot (not shown), giving a K_m of 8 μM and a V of 10 pmol · μg^{-1} DNA · h^{-1}.

the amount of radiolabeled tracer was kept constant, and increasing amounts of non-radioactive L-acetylcarnitine was added in determining K_m and V. Distribution of the radioactivity among carnitine and acylcarnitines after incubation with L-[*Me*-^{3}H]acetylcarnitine, showed 60% as carnitine and 40% as acetylcarnitine. This is quite similar to that previously reported after incubation with L-[*Me*-^{3}H]carnitine [4]. It is therefore likely that radiolabeled acetylcarnitine rapidly mixes with the intracellular carnitine pool.

When the uptake of L-[*Me*-^{3}H]acetylcarnitine was inhibited by L-carnitine (not shown) K_i for the latter was 4 μM, and there was no change in V. The K_i of L-acetylcarnitine on the uptake of L-[*Me*-^{3}H]carnitine was 8 μM (not shown). These data indicate a competitive type of inhibition between L-carnitine and L-acetylcarnitine. The inhibition (98%) of L-acetylcarnitine uptake of 1 mM *N*-ethylmaleimide (not shown), is similar to the inhibition found for the uptake of L-carnitine (Table I), and further supports the assumption of a common transport mechanism for carnitine and acetylcarnitine.

The studies of carnitine turnover time in rats have given different results for radiolabeled DL- and L-carnitine [17,18]. In addition there have been discrepancies between elimination of carnitine as calculated from kinetic data and the measurements of urinary excretion of carnitine [19,20]. Since we have found that D-carnitine inhibits the transport of L-carnitine, it is apparent that DL- and L-carnitine tracers would give different results. It is possible that L-acetylcarnitine, present in rat plasma [10], and transported by the same mechanism as L-carnitine, could at least partly be responsible for such discrepancies.

The transport mechanism described for L-carnitine and L-acetylcarnitine could be the physiological basis for the high carnitine concentration in the heart in vivo [4]. It is possible that a defect in this cencentrative mechanism could be involved in the pathogenesis in some diseases in which the concentration of carnitine in the heart is known to be low [5,6,7].

Acknowledgements

We are indebted to Åke Bjørke for skilled technical assistance. This study was supported by a grant from The Norwegian Council on Cardiovascular Diseases.

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