Biochimica et Biophysica Acta, 735 (1983) 197–202
Elsevier

BBA 71876

EFFECT OF *N*-ETHYLMALEIMIDE ON LEUCINE TRANSPORT IN THE CHANG LIVER CELL

II. EFFECT ON THE KINETICS OF Na^+-INDEPENDENT TRANSPORT

TSUNEO TAKADERA and TETSURO MOHRI

Department of Physiological Chemistry, Hokuriku University School of Pharmacy, Kanazawa 920-11 (Japan)

(Received June 20th, 1983)

Key words: Leucine transport; N-Ethylmaleimide; Dinitrophenol; Membrane perturbation; (Chang liver cell)

The Na^+-independent leucine transport system is resolved into two components by their different affinity (K_m about 44 μM and 8.0 mM) for leucine in the Chang liver cell. Treatment of the cells with *N*-ethylmaleimide (1 mM) specifically stimulates the high-affinity component of the Na^+-independent system by greatly increasing its V_{max} value, whereas the V_{max} value of the low-affinity component is markedly lowered. The stimulatory effect of *N*-ethylmaleimide on leucine transport is reduced by prior treatment of the cells with 2,4-dinitrophenol, but this phenomenon seems to be irrelevant to the ATP-depleting action of the uncoupler. The treatment with 2,4-dinitrophenol has been found not to be inhibitory on the subsequent Na^+-independent leucine uptake itself. Treatment with dibucaine, a phospholipid-interacting drug, also reduces to varying degrees (depending on its concentration) the stimulatory effect of *N*-ethylmaleimide on the subsequent leucine uptake, although pretreatment with dibucaine can stimulate the Na^+-independent leucine uptake itself. We conclude that the stimulatory effect of *N*-ethylmaleimide on leucine transport is not correlated with the energy level of cell, but involves the perturbation of the membrane bilayer structures.

Introduction

The effects of various SH reagents such as *N*-ethylmaleimide, *p*-chloromercuribenzoate, *p*-chloromercuriphenyl sulfonate and iodoacetate on the active transport of amino acids have been studied in order to elucidate the necessity for SH groups for the activity of the cell membrane transport systems [1–6].

We reported previously that *N*-ethylmaleimide can stimulate specifically Na^+-independent leucine uptake, while *p*-chloromercuriphenyl sulfonate inhibits this uptake in the Chang liver cell [7]. The effect of *N*-ethylmaleimide, however, was found to be dependent on the concentration of leucine, variable in higher concentrations. In the present work, the stimulatory effect of *N*-ethylmaleimide on the Na^+-independent leucine uptake in the Chang liver cell has been characterized kinetically over a wide range of leucine concentrations and relations of the effect of *N*-ethylmaleimide to the energy level of the cell and the membrane lipid structures have been discussed.

Materials and Methods

Chemicals. L-[U-^{14}C]Leucine was purchased from Amersham International, U.K. *N*-Ethylmaleimide was purchased from Sigma. Dibucaine hydrochloride (Percamin) was obtained by the courtesy of Teikoku Kagaku Ind. Co., Osaka, Japan. Other reagents used here were of special grade from Wako Pure Chemicals Co., Tokyo.

Cells. Chang liver cells were cultured as monolayers in polystyrol petri dishes (35 or 60 mm), multi-well plates (24 wells each, 17 mm) or glass

bottles (80 × 140 mm) with 45% (v/v) Eagle's minimum essential medium [8], supplemented with 45% (v/v) Hanks' salts solution containing 0.4% (w/v) lactalbumin hydrolysate and 10% (v/v) bovine serum, under 5% CO_2 in air at 37°C.

Measurement of amino acid uptake. In this study we used two methods of amino acid transport assay. One of them was the same as described previously [7]: incubation for measurement of amino acid uptake was carried out for 1–2 min at 37°C with cells grown as monolayers in petri dishes (35 mm). The other, only used in kinetic experiments, employed cells grown as monolayers in multi-well plates according to the procedure described by Gazzola et al. [9]. Incubation for measurement of amino acid uptake in this method was carried out for 30 s at 37°C in the medium in which the NaCl and sodium phosphate of Krebs-Ringer phosphate buffer were replaced by equimolar concentrations of choline chloride and potassium phosphate, respectively, and 0.1% glucose (as specified in the results). This substitute of Krebs-Ringer phosphate is referred to as choline medium. Incubation medium contained labelled and non-labelled L-leucine of desired total concentrations (Results). Pretreatment of cells with *N*-ethylmaleimide (1 mM), dibucaine (0.25–1 mM), and 2,4-dinitrophenol (0.5–8 mM) was performed in choline medium at 37°C for periods as specified in the results. At the conclusion of the uptake period, the medium was removed by inverting the plate and transport was terminated by two 1-ml washes with ice-cold medium. After removal of the residual medium, 220 μl of 5% (w/v) trichloroacetic acid was added to each well and after a 30-min incubation at room temperature, 200 μl of this extract was dried on a glass filter (Whatman GF/B, 24 mm) and counted for radioactivity using conventional toluene scintillation fluid. Cell proteins of each well were dissolved with 1 M NaOH overnight and subjected to protein determination.

Determination of intracellular free amino acid concentrations. For this analysis, cells were grown as monolayers in several glass bottles for each group. After the preincubation with choline medium for 30 min at 37°C, the cells were incubated with the same medium in the presence or absence of *N*-ethylmaleimide (1 mM). Then the cells were quickly washed with ice-cold medium. After addition of 70% ethanol, the cells were harvested with a rubber policeman. The mixture of cellular suspension and rinse solution was centrifuged for 10 min at 3000 × *g*. The supernatant fluid was evaporated and redissolved in a small volume of 3% 5-sulfosalicylic acid. Samples were incubated for 5 min in a boiling water bath and after cooling centrifuged for 10 min at 12000 × *g*. An aliquot of the supernatant was applied on a Hitachi amino acid analyzer (model KLA-5) for determination of amino acids. The precipitate from ethanol solution mentioned above was subjected to protein determination.

Determination of cellular ATP content. Cellular ATP content after various treatments of the cells was measured as described previously [10]. The cells attached to 60-mm petri dishes were rapidly washed twice with ice-cold choline medium. The cell sheets of each dish were scraped off with a rubber policeman into 5 ml cold 6% $HClO_4$ in a centrifuge tube. The supernatant fraction obtained by centrifugation at 3000 × *g* was neutralized with K_2CO_3 solution to remove $HClO_4$ as $KClO_4$. An aliquot of the final clear solution was used for determination of ATP with an ATP-luminescence reader from Aloka Co. using fire-fly lanterns. The precipitate fraction was subjected to protein determination.

Protein determination. Cellular protein was determined by the method of Lowry et al. [11] using bovine serum albumin as standard.

Results

Effect of N-ethylmaleimide on the kinetic parameters of leucine transport in choline medium

We reported previously that *N*-ethylmaleimide increased specifically the Na^+-independent uptake of leucine by the cell. In this experiment, 30 s uptake of leucine was measured over a leucine concentration range of 0.01–10 mM in choline medium to estimate the kinetic parameters of Na^+-independent leucine transport and the effect of pretreatment with *N*-ethylmaleimide on them. The time-course of leucine uptake was linear for at least 45 s over this concentration range. Fig. 1 shows the Eadie-Hofstee plot of the rate of Na^+-independent leucine uptake of the cells after incubation with or without *N*-ethylmaleimide (1 mM)

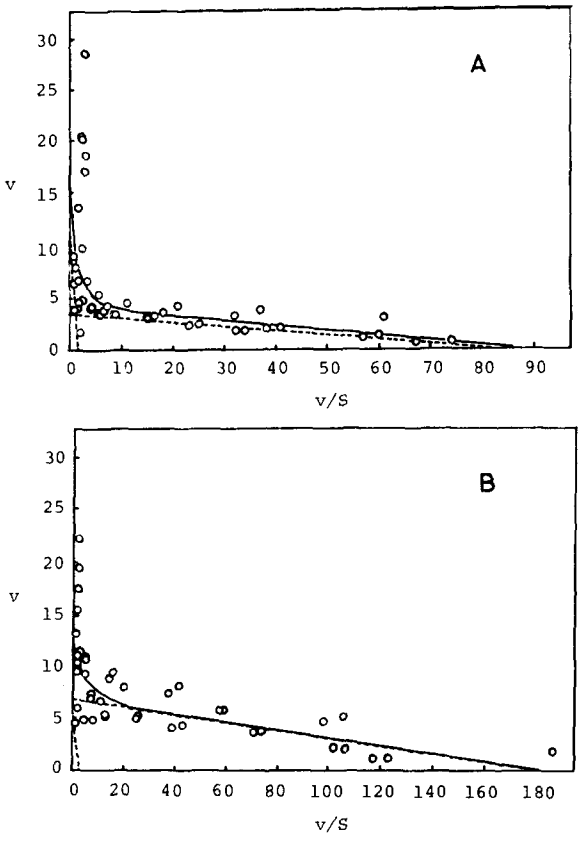

Fig. 1. Effect of *N*-ethylmaleimide on the kinetics of leucine transport in choline medium. Cells grown on multi-well plates were incubated for 30 s in choline medium containing 0.1% glucose and various concentrations of labelled leucine in a range 0.01–10 mM after incubation for 10 min in the presence (B) or absence (A) of *N*-ethylmaleimide. The uptake by the cells of leucine was plotted according to the method of Eadie using v (nmol/min per mg protein) and S (mM). The solid lines show the computer-derived best fits for the plots, and the dashed lines show the intercept values after resolution of the two components for each curve. The nonsaturable component of uptake was subtracted.

for 10 min. It has been revealed that the leucine transport consists of at least two components kinetically distinguished in control and treated cells. As shown in Table I, the V_{max} value of the high-affinity component was shown to increase markedly after *N*-ethylmaleimide treatment, while that of the low-affinity component was greatly reduced. The affinity of leucine for the high-affinity component was not changed by *N*-ethylmaleimide treatment, while that of the low-affinity component increased.

TABLE I

THE KINETIC PARAMETERS OF LEUCINE TRANSPORT AFTER INCUBATION WITH OR WITHOUT *N*-ETHYLMALEIMIDE

The values were calculated from the data shown in Fig. 1. See the legend to Fig. 1 for experimental conditions.

Component	Control		*N*-Ethylmaleimide	
	K_m (mM)	V_{max} (nmol/min per mg protein)	K_m (mM)	V_{max} (nmol/min per mg protein)
High-affinity	0.044	3.8	0.040	7.0
Low-affinity	8.0	12	4.0	6.0

Effect of N-ethylmaleimide on the free amino acid contents

Cells were routinely depleted of internal amino acids by 30 min incubation in amino-acid-free medium prior to uptake measurements to minimise the trans-effect. In a previous paper [7] we showed that pretreatment of cells with *N*-ethylmaleimide slightly decreased the efflux of leucine. Therefore we examined the pool of free amino acids to check the possibility that the difference of the changes in the size of the system L-reactive amino acid pool during the time of preincubations

TABLE II

FREE AMINO ACID CONTENTS IN CELLS AFTER INCUBATION WITH OR WITHOUT *N*-ETHYLMALEIMIDE

Cells grown in bottles were incubated for 30 min at 37°C with choline medium and then incubated for additional 10 min in the presence or absence of 1 mM *N*-ethylmaleimide. Amino acids were extracted and assayed as described in Materials and Methods. In the case of methionine, less than 0.1 nmol/mg protein was present.

Amino acid	Contents (nmol/mg protein)	
	Control	*N*-Ethylmaleimide
Valine	0.7	0.7
Isoleucine	0.3	0.3
Leucine	0.9	0.6
Tyrosine	0.4	0.3
Phenylalanine	0.5	0.6
Histidine	0.4	0.2
Methionine	–	–
Total system L-amino acids	3.2	2.7

with and without *N*-ethylmaleimide would cause the apparent increase of the rate of inward leucine transport by trans-stimulation. As shown in Table II, the intracellular levels of seven amino acids that are assumed to be transported by system L were not increased by *N*-ethylmaleimide treatment.

Effect of N-ethylmaleimide on leucine uptake after treatment of cells with 2,4-dinitrophenol

The effect of *N*-ethylmaleimide on the uptake of leucine at 0.17 mM was measured in choline medium in the absence of glucose after treatment with various concentrations of 2,4-dinitrophenol for 30 min (Table III). The stimulation by *N*-ethylmaleimide was greatly reduced after 2,4-dinitrophenol treatment, but some stimulation remained even at 8 mM dinitrophenol. 2,4-Dinitrophenol had no significant effect on the leucine uptake itself (−NEM column in Table III).

Change of the kinetic parameters of N-ethylamleimide after 2,4-dinitrophenol treatment

The effect of *N*-ethylmaleimide on the kinetic parameters of leucine uptake by the cells was measured in choline medium after treatment with 2,4-dinitrophenol (2 mM) for 30 min (Table IV).

TABLE III

EFFECT OF *N*-ETHYLMALEIMIDE ON LEUCINE UPTAKE IN CHOLINE MEDIUM AFTER TREATMENT OF CELLS WITH VARIOUS CONCENTRATIONS OF 2,4-DINITROPHENOL

Dishes of cells were incubated with labelled leucine (0.17 mM) in choline medium for 2 min after treatment of cells for 30 min with varying concentrations of 2,4-dinitrophenol, and then incubated for additional 10 min in the presence or absence of *N*-ethylmaleimide (NEM) in choline medium containing no glucose. Each value represents the mean and the standard error of triplicate determinations.

2,4-Dinitrophenol concentration (mM)	Leucine uptake		
	nmol/min per mg protein		Stimulation by NEM (%)
	−NEM	+NEM	
0	1.21 ± 0.10	2.76 ± 0.03	128
0.5	1.12 ± 0.07	1.58 ± 0.09	40
2	1.20 ± 0.06	1.73 ± 0.11	44
4	1.11 ± 0.05	1.58 ± 0.05	42
8	1.04 ± 0.01	1.34 ± 0.11	30

TABLE IV

EFFECT OF *N*-ETHYLMALEIMIDE ON THE KINETIC PARAMETERS OF LEUCINE TRANSPORT AFTER 2,4-DINITROPHENOL TREATMENT OF CELLS

Cells grown on multi-well plates were incubated with 1 mM *N*-ethylmaleimide for 10 min after treatment with 2 mM 2,4-dinitrophenol for 30 min and then incubated with various concentrations (0.01–10 mM) of labelled leucine in choline medium for 30 s. Kinetic constants were calculated for each component as described in the legend to Fig. 1.

Component	Control		*N*-Ethylmaleimide	
	K_m (mM)	V_{max} (nmol/min per mg protein)	K_m (mM)	V_{max} (nmol/min per mg protein)
High-affinity	0.040	3.5	0.047	4.5
Low-affinity	8.0	11	4.0	9.0

The V_{max} of the high-affinity component was increased 30% by *N*-ethylmaleimide pretreatment, the increase being smaller that in the cells not treated with 2,4-dinitrophenol. The K_m change of the low-affinity component was closely comparable between 2,4-dinitrophenol-treated and -untreated cells.

Effect of N-ethylmaleimide and 2,4-dinitrophenol on cellular ATP content

To investigate the relations of the effect of *N*-ethylmaleimide pretreatment on leucine uptake and that of 2,4-dinitrophenol on the stimulation of leucine uptake by *N*-ethylmaleimide to the change of cellular ATP content, the content was determined after incubation of cells with or without *N*-ethylmaleimide for 10 min and after successive incubations with 2 mM 2,4-dinitrophenol for 10 min and then with *N*-ethylmaleimide in choline medium in the presence or absence of glucose (Table V). The treatment with *N*-ethylmaleimide decreased the ATP content, irrespective of the presence or absence of glucose, when 2,4-dinitrophenol treatment was omitted. The effect of *N*-ethylmaleimide was absent or very slight after 2,4-dinitrophenol treatment.

Effects of dibucaine on leucine uptake and on the stimulation by N-ethylmaleimide

Dibucaine is known to interact with the phos-

TABLE V

EFFECTS OF *N*-ETHYLMALEIMIDE AND 2,4-DINITROPHENOL ON CELLULAR ATP CONTENT

Dishes of cells were incubated at 37°C for 10 min with or without 2 mM 2,4-dinitrophenol (DNP), and then incubated for additional 10 min in the presence or absence of 1 mM *N*-ethylmaleimide (NEM) with or without 0.1% glucose. All incubations were carried out using choline medium. Determination of cellular ATP was performed as described in Materials and Methods. Each value represents the mean of three experiments and the standard error.

Glucose in medium	Treatment		ATP content (nmol/mg protein)
	DNP	NEM	
–	–	–	0.83 ± 0.17
		+	0.05 ± 0.02
+	–	–	0.71 ± 0.08
		+	0.45 ± 0.10
–	+	–	0.11 ± 0.01
		+	0.11 ± 0.03
+	+	–	0.54 ± 0.12
		+	0.42 ± 0.05

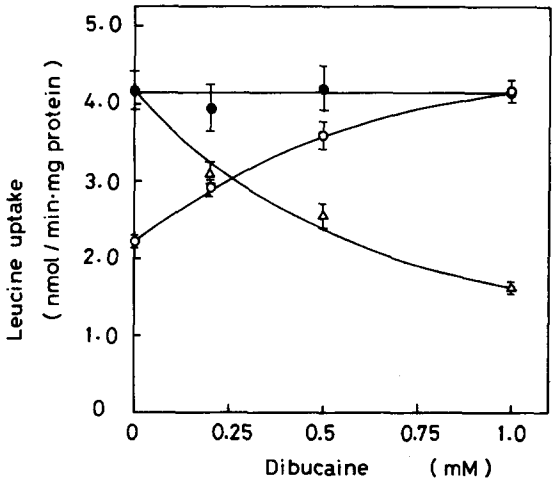

Fig. 2. Effects of treatment with dibucaine on leucine uptake and on the stimulation by *N*-ethylmaleimide. Dishes of cells were preincubated with *N*-ethylmaleimide for 10 min after (△) or before (●) treatment with dibucaine (approx. 1 mM) for 10 min and incubated with labelled leucine (0.17 mM) for 1 min in choline medium containing 0.1% glucose. The single effect of dibucaine was measured after treatment with varying concentrations of dibucaine for 10 min (○). Each point represents the mean ± S.E. of triplicate determinations.

pholipid components of the cell membrane [12]. Treatment of cells with this drug increased the subsequent leucine uptake in choline medium to varying extents depending on its concentration (approx. 1 mM) (Fig. 2). But this effect was shown either not to be additive to that of preceding *N*-ethylmaleimide treatment or paradoxically to be reversed by subsequent *N*-ethylmaleimide treatment; the higher the concentration of dibucaine, the larger both the depression of the effect of *N*-ethylmaleimide and the stimulation of the uptake by dibucaine itself.

Discussion

It has been demonstrated in the present work that the Na^+-independent system for leucine transport in the Chang liver cell can be resolved into two components by their different affinity for leucine.

Recently it has been revealed by Weissbach et al. [13,14] that the primary culture of rat hepatocytes has two Na^+-independent transport systems (L1 and L2) different in affinity for leucine and other branched-chain amino acids. The K_m values for leucine in systems L1 and L2 of the rat hepatocytes are proposed to be 7 μM and 0.98 mM in the early stages of culture, respectively – very low compared to those of the high- and low-affinity components in the Chang liver cell. It is also reported there that the K_m values as well as the V_{max} values of L1 and L2 change more-or-less with time of culture.

The high-affinity component of the Na^+-independent system of the Chang liver cell is specifically stimulated by pretreatment of cells with 1 mM *N*-ethylmaleimide (Fig. 1 and Table I). The stimulation of the high-affinity component was found to be due to an increase in its V_{max}. The low-affinity component was depressed in its V_{max} by *N*-ethylmaleimide pretreatment, while its apparent K_m value was slightly reduced. The finding showing that treatment with *N*-ethylmaleimide does not essentially affect the intracellular levels of system L-reactive amino acids in the experimental conditions (Table II) proposes that the stimulatory effect of *N*-ethylmaleimide is not elicited by trans-stimulation.

Prior treatment with 2,4-dinitrophenol at con-

centrations of 0.5 mM or more reduced the stimulatory effect of *N*-ethylmaleimide on the leucine uptake, irrespective of the presence (data not shown) or absence (Table III) of glucose in incubation medium. This effect of 2,4-dinitrophenol was explicit in the change of the V_{max} value of the high-affinity component of leucine transport (Table IV). It has been demonstrated that the stimulatory effect of *N*-ethylmaleimide on the high-affinity component of leucine transport does not reflect any increase of intracellular ATP level. Neither can the effect of treatment with 2,4-dinitrophenol on the stimulation of *N*-ethylmaleimide be correlated with the change of cellular ATP content (Table V). Sheetz et al. [15] reported that 2,4,6-trinitrophenol, which is not an uncoupling agent, affected the shape of human erythrocytes, as did 2,4-dinitrophenol, and proposed that these two agents have a direct effect on the lipid bilayer of the cell membrane, a differential expansion or contraction of the two half-layers [15].

In this connection, a particular membrane perturbation is expected to be involved in the stimulatory effect of *N*-ethylmaleimide. Dibucaine, thought to have an interaction with the membrane phospholipids and affect a variety of cell activities [16–18], was realized to be capable of increasing leucine uptake to varying extents depending on its added concentration (Fig. 2). Prior treatment with dibucaine, however, impaired, completely at 1.0 mM, the stimulatory effect of *N*-ethylmaleimide (1 mM) on the uptake. Dibucaine (approx. 1 mM) did not disturb or enhance the stimulatory effect of *N*-ethylmaleimide, when used after *N*-ethylmaleimide. These results may suggest that a prerequisite for the stimulatory effect of *N*-ethylmaleimide on leucine uptake is a definite lipid structure of the cell membrane that can be destroyed or modified by treatment with dibucaine to convert *N*-ethylmaleimide into a depressor of leucine uptake by the high-affinity component; depression is supposed to be in correlation with the extent of modification. It seems that the effect of *N*-ethylmaleimide on the high-affinity component of Na^+-independent leucine transport in the Chang liver cell involves alteration of the properties of protein-lipid interaction related to the transport system in the cell membrane through the blocking of SH groups of some cellular proteins.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Scientific Research (No. 00557521) from the Ministry of Education, Science and Culture, Japan.

References

1 Janick, P.A., Grunwald, G.B. and Wood, J.M. (1977) Biochim. Biophys. Acta 464, 328–337
2 Kaback, H.R. and Patel, L. (1978) Biochemistry 17, 1640–1646
3 Anraku, Y. (1968) J. Biol. Chem. 243, 3128–3135
4 Young, J.D. (1980) Biochim. Biophys. Acta 602, 661–672
5 Nelson, S., Glover, G.I. and Magill, C.W. (1975) Arch. Biochem. Biophys. 168, 483–489
6 Reiser, S. and Christiansen, P.A. (1971) Biochim. Biophys. Acta 225, 123–139
7 Takadera, T. and Mohri, T. (1982) Biochim. Biophys. Acta 691, 293–299
8 Eagle, H. (1959) Science 130, 432–437
9 Gazzola, G.C., Dall'Asta, V., Franchi-Gazzola, R. and White, M.F. (1981) Anal. Biochem. 115, 368–373
10 Mohri, T., Yamaguchi, T. and Kitagawa, H. (1979) J. Biochem. (Tokyo) 85, 681–689
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
12 Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 265, 169–186
13 Weissbach, L., Handlogten, M.E., Christensen, H.N. and Kilberg, M.S. (1982) J. Biol. Chem. 257, 12006–12011
14 Handlogten, M.E. and Weissbach, L. and Kilberg, S. (1982) Biochem. Biophys. Res. Commun. 104, 307–313
15 Sheetz, M.P., Painter, R.G. and Singer, S.J. (1976) J. Cell Biol. 70, 193–203
16 Papahadjopoulos, D., Jacobson, K., Poste, G. and Shepherd, G. (1975) Biochim. Biophys. Acta 394, 504–519
17 Poste, G., Papahadjopoulos, D., Jacobson, K. and Vail, W.J. (1975) Biochim. Biophys. Acta 394, 520–539
18 Gazitt, Y., Loyter, A. and Ohad, I. (1977) Biochim. Biophys. Acta 471, 361–371