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STIMULATORY EFFECT OF GLUCAGON AND DIBUTYRYL-cAMP SPECIFICALLY ON THE Na^+-INDEPENDENT AMINO ACID TRANSPORT OF CHANG LIVER CELL

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Pretreatment(2 h) of Chang liver cells with glucagon and dibutyryl-cAMP has been shown to stimulate specifically the Na^+-independent transport system for amino acids. The stimulation of Na^+-independent leucine uptake was completely or largely inhibited by prior incubation(30 min)of the cells with colchicine or cytochalasin B. Glucagon treatment colud only change the Vmax value of the high-affinity component of Na^+-independent leucine transport and the apparent Km value of the low-affinity one, whereas dibutyryl-cAMP treatment more or less affected all of the kinetic constants of both of the two components in favor of promotion of leucine tansport. These results of kinetic analysis indicate that dibutyryl-cAMP may not perfectly play the role of glucagon in activation of the Na^+-independent transport system for leucine.

Glucagon has been widely recognized to be an activator of adenyl cyclase in liver(1). It has been demonstrated recently that pretreatment of hepatic cells (2-7) or slices (8) or animals (9,10) with the hormone, singly or in combination with dexamethasone(5) or theophylline(9), stimulates the subsequent uptake by the cells, slices or livers(with and without hepatoma) of amino acids, 2-aminoisobutyrate(2-6, 8-10), cycloleucine (8) and alanine(7). The stimulation elicited by glucagon was synchronous with or preceded by increase of cellular cAMP concentration(5,11) and mimicked by addition of dibutyryl-cAMP to incubation medium(3,5,6,8,12) or injection of it into rats (10).

We have revealed in the present work with Chang liver cell, a human culture cell, that both glucagon and dibutyryl-cAMP are capable of producing differential effects on amino acid transport systems and that an efficient emergence of the stimulatory effect of them specifically on Na^+-independent leucine transport system appears to require prerequisitely complete functioning of the cytoskeleton systems.

Abbreviations: cAMP, cyclic AMP; Bu_2cAMP, dibutyryl-cAMP; KRP, Krebs-Ringer phosphate buffer; AIB, 2-aminoisobutyric acid.

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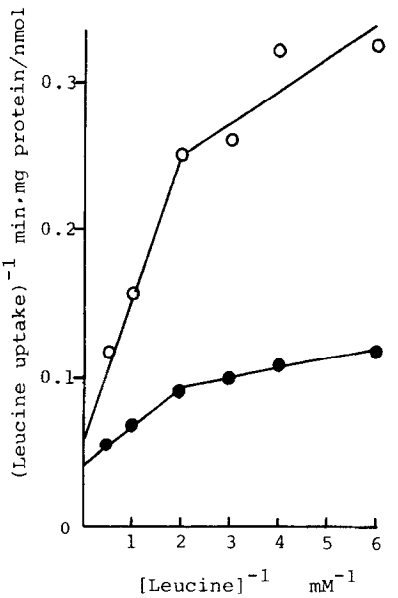

Fig. 1 Kinetic analysis of the stimulatory effect of glucagon on Na^+-independent leucine transport. Preincubation with glucagon(1.5×10^{-7} M) for 2 h was carried out in KRP at 37° C and then the uptake(1 min) of leucine in a range from 0.17 to 2 mM was measured in choline medium. Lineweaver-Burk plots of the values(means of triplicated determinations) are shown for control(○) and glucagon(●) groups.

Materials and Methods

Chang liver cells were grown as monolayers on plastic culture dishes of 35 mm in diameter for several days with a medium consisting of Eagle minimum essential medium[13] supplemented with 0.4%(w/v) lactalbumin hydrolysate and 10%(v/v) bovine serum. Pretreatment of cells with the agents specified in the results and incubation for measurement of glycine uptake were all performed in Krebs-Ringer phosphate buffer(KRP) supplemented with 0.1%(w/v) glucose at 37 °C. Incubation for measurement of leucine uptake was done at 37 °C in a medium, designated choline medium, where equimolar concentrations of choline chloride and K_2HPO_4 were substituted for NaCl and Na_2HPO_4 of KRP, respectively, unless otherwise mentioned. The uptake of amino acids was measured by the procedures described previously [14], except for adopting a shorter incubation period(1 min) throughout the experiments. After each preincubation the cell layers and the inside of dishes were rinsed twice with cold choline medium. Labeled amino acids used were [U-^{14}C]glycine and L-[U-^{14}C]leucine purchased from Amersham. Glucagon was purchased from Sigma, dibutyryl-cAMP from Böhringer, cytochalasin B from Aldrich, and colchicine and cycloheximide from Wako Pure Chemical Industries, Japan.

Results and Discussion

Treatment of cells with glucagon(1.5×10^{-7} M) for 2 h stimulated the subsequent Na^+-independent uptake of labeled leucine over the range of concentrations from 0.17 to 2 mM(Fig. 1). The kinetic analysis of leucine uptake has revealed that the Na^+-independent transport of leucine consists of two components different in both Vmax and apparent Km values and that glucagon

Table I

Effect of pretreatment with glucagon on leucine uptake and inhibition of it by colchicine and cytochalasin B

Preincubation I (30 min)	Preincubation II (2 h)	Uptake (1 min)	% Increase	P
———	———	4.15±0.45		
———	Glucagon	6.67±0.04	61	<0.01
Colchicine	———	4.20±0.20	1	n.s.
Cytochalasin B	———	4.26±0.40	3	n.s.
Colchicine	Glucagon	4.48±0.49	8	n.s.
Cytochalasin B	Glucagon	4.67±0.19	13	n.s.

Preincubation with or without(—) colchicine(10^{-6} M), cytochalasin B (10^{-6} M) and glucagon(1.5 x 10^{-7} M) was performed at 37 °C for periods specified in the table in KRP and then the uptake of leucine(0.17 mM) was measured in choline medium. The uptake is expressed as nmol/min per mg protein (mean of triplicated determinations ± S.E.M.).
n.s.: Not significant.

pretreatment increases the Vmax value(10.3 nmol/min per mg protein vs. 3.6 nmol/min per mg protein for control) of the high-affinity component with no effect on its apparent Km value and reduces only the apparent Km value(0.90 mM vs. 1.97 mM for control) of the low-affinity component. The stimulation of leucine uptake due to glucagon pretreatment was completely abolished by the treatment of the cells with cytochalasin B(10^{-6} M) or colchicine(10^{-6} M) for 30 min prior to glucagon(Table I), while treatment with either of these inhibitors of cytoskeleton formation had no significant effect on the basal uptake of leucine. Pretreatment of cells with glucagon had no effect on the uptake of glycine(0.33 mM) in KRP(data not shown).

Table II
Change of the kinetic constants of the high- and low-affinity components of leucine transport by pretreatment with dibutyryl-cAMP

	Km (mM)		Vmax(nmol/min per mg protein)	
	High-affinity	Low-affinity	High-affinity	Low-affinity
Control	0.13	1.85	5.69	18.1
Bu_2cAMP	0.05	1.55	9.64	26.1

Preincubation with or without(control) dibutyryl-cAMP(0.2 mM) was performed for 2 h in KRP at 37°C and then leucine uptake was measured in leucine concentrations ranging from 0.17 to 2 mM in choline medium. Kinetic constants were calculated for each group from the Lineweaver-Burk plot of the values of the uptake(1 min) according to the two-component analysis method of Neal (20).

Table III

Effect of pretreatment with dibutyryl-cAMP on leucine and glycine uptakes

Amino acid 0.33 mM	Medium*	Uptake(nmol/min per mg protein) Control	Bu_2cAMP	% increase	P
Leucine	KRP	8.82±0.10	10.9 ±0.30	24	<0.01
	Choline	5.87±0.68	9.81±0.17	67	<0.01
Glycine	KRP	14.2 ±0.39	13.6 ±0.86	-4	n.s.

*Medium used for measurement of amino acid uptake(1 min; KRP, Krebs-Ringer phosphate buffer; Choline, choline medium).

Preincubation with or without(control) dibutyryl-cAMP was performed in KRP for 2 h at 37°C for all amino acids. The values of uptake are expressed as mean of triplicated determinations ±S.E.M.
n.s.: Not significant.

Pretreatment of cells with dibutyryl-cAMP(0.2 mM) for 2 h also stimulated the Na^+-independent uptake of leucine at various concentrations, changing the kinetic parameters of the two components of leucine transport as summarized in Table II. As shown in Table III the stimulation of leucine uptake by dibutyryl-cAMP was apparently much smaller in KRP than in choline medium when they were employed as incubation medium for uptake measurement. Glycine uptake in KRP was not affected by a comparable dibutyryl-cAMP pretreatment.

The stimulation of Na^+-independent leucine uptake was largely diminished, whether significantly or not, by the prior treatment of cells with colchicine and cytochalasin B(Table IV).

Table IV

Effect of pretreatment with dibutyryl-cAMP on leucine uptake and inhibition of it by colchicine and cytochalasin B

Preincubation I 30 min	Preincubation II 2 h	Uptake 1 min	% increase	P
———	———	3.52±0.10		
———	Bu_2cAMP	5.31±0.46	51	<0.05
Colchicine	———	3.81±0.09	8	n.s.
Cytochalasin B	———	3.95±0.84	12	n.s.
Colchine	Bu_2cAMP	4.24±0.06	20	<0.01
Cytochalasin B	Bu_2cAMP	4.07±0.27	16	n.s.

Preincubation with or without(—) colchicine(10^{-6} M), cytochalasin B (10^{-6} M) and dibutyryl-cAMP(0.2 mM) was performed at 37 °C for periods specified in the table in KRP and then the uptake of leucine(0.17 mM) was measured in choline medium. Expressions of the data are similar to those in Table I.

To check a possibility of increased exchange of labeled leucine with intracellular amino acids that might be increased as a consequence of promoted proteolysis induced by glucagon 15 , the pool of free amino acids within the cells was determined by the method of ninhydrin reaction using leucine as standard with the trichloroacetate extracts of control(incubated for 2 h in KRP) and glucagon-treated cells. Free amino scids in cells were 0.408 ±0.04(S.E.M. of four determinations) μmol leucine equivalent/mg protein in total after 2 h of glucagon treatment, compared with 0.393 ± 0.05 μmol leucine equivalent/mg protein in control cells.

The stimulatory effect of dibutyryl-cAMP on Na^+-independent leucine transport was not affected by pretreating cells simultaneously with cycloheximide(0.5 mM) for 2 h(data not shown).

It has been demonstrated in the present work that Na^+-independent amino acid transport is specifically activated by pretreatment with glucagon or dibutyryl-cAMP in Chang liver cell; glycine uptake, which has proved to be Na^+-dependent to a large proportion[16], was not responsive to pretreatment of cells with these agents when determined in KRP. Leucine is transported across the cell membrane by both Na^+-dependent and -independent systems when the cell is incubated in Na^+-repleted medium, e.g. KRP[16]. In fact the stimulatory effect of those agents on leucine uptake was rather attenuated in KRP used as incubation medium(Table III). Stimulation of amino acid uptakes, e.g. those of 2-aminoisobutyric acid, cycloleucine and alanine, by glucagon treatment and incubation with cAMP or its active analogue was so far reported mostly with Na^+-repleted medium as assay medium in various cell species. Those amino acids, however , have been shown to have overlapping affinity more or less for both system A, a Na^+-dependent system, and system L, a Na^+-independent system, participating in amino acid transport across the plasma membrane in cells [16-19], while glycine very preferentially reacts with the former system [16-18]. Therefore it may be doubtful to rationalize that those stimulators have an exclusive effect on system A as insisted by many.

Preincubation of cells for periods less than 30 min with glucagon or dibutyryl-cAMP was not enough for the emergence of their effect on leucine

uptake(data not shown). On the other hand the presence of the inhibitor of protein synthesis throughout 2 h of preincubation had no action on the induction of the stimulatory effect of dibutyryl-cAMP. It is thence highly postulated that the effect of these two agents on leucine transport involves as an intermediate, rate-limiting step a phosphorylation of some population of cellular protein that is not inducibly synthesized on addition of the agents. In the rat liver slices[8] and cells[7] the stimulation of AIB uptake by pretreatment with cAMP for about 100 or 120 min was impaired by the simultaneous treatment with cycloheximide.

These results may suggest that there is a species difference in the mechanism of action of cAMP and its analogue on amino acid transport systems. Based on the data of the kinetic analyses of the stimulatory effect of glucagon and dibutyryl-cAMP on leucine transport in the present work(compare the data presented in the explanation of Fig. 1 in the text with those shown in Table II), the modes of actions of these two agents on leucine transport are not considered perfectly identical in the cell. Nevertheless the effects of glucagon and dibutyryl-cAMP on the uptake of leucine(0.17 mM) were never additive when the two agents were simultaneously added to preincubation(2 h) medium(data not shown).

The inhibitory effect of colchicine and cytochalasin B, both of which interfer with functioning of the cytoskeleton, only on the stimulation of Na^+-independent transport of leucine by glucagon and dibutyryl-cAMP, not manifesting itself on the basal uptake of leucine(Tables I and IV), may imply that complete cytoskeleton structures are prerequisite or greatly influential for the emergence of the stimulatory effect of glucagon and cAMP on Na^+-independent leucine transport, but not for the basic mechanism of the transport. these results are consistent with those of Prentki et al., who have shown in rat hepatocytes that glucagon-induced promotion of AIB uptake can be impaired by 40% by previous treatment of the cells with colchicine, while control uptake is hardly affected by the treatment[2].

The results reported in this communication suggest a regulation of the transport of branched-chain amino acids across the cell membrane by organized cellular systems activated by glucagon action mediated, at least partly, by cAMP in human liver cell.

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