GLUCAGON STIMULATES THE A SYSTEM FOR NEUTRAL AMINO ACID TRANSPORT IN ISOLATED HEPATOCYTES OF ADULT RAT.

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SUMMARY : The effect of glucagon on neutral amino acid transport systems was studied in freshly-prepared suspensions of isolated hepatocytes by measuring basal and hormone-stimulated α-aminoisobutyric acid (AIB) and cycloleucine uptake. Glucagon stimulated AIB uptake against a concentration gradient. Half-maximal stimulation occured with 0.4 nM glucagon. Glucagon stimulation was restricted to the A mediation of transport. The effect was rapid, occured without time lag, was abolished by cycloheximide and mimicked by dibutyryl cyclic AMP.

Amino acid transport in the liver has been investigated *in vitro* mainly with the use of slices (1,2) or of the perfused organ (3,4). It has been difficult, with these techniques, to characterize the transport systems and to assign hormone effects to a given transport system. Although isolated hepatocytes may represent a convenient material for such studies, it has recently been stressed (5) that freshly-prepared suspensions of hepatocytes fail to actively transport the nonmetabolizable analog α-aminoisobutyric acid (AIB). However, it has been also reported in brief form (6) that AIB can be concentrated by these cells.

We observed that freshly-isolated hepatocytes can indeed actively transport neutral amino acids and we characterized the transport systems involved (7) using a methodology similar to that employed by Christensen (8) with the Ehrlich ascites tumor cell. The data presented in this communication indicates that glucagon specifically stimulates the A transport system in freshly-prepared suspensions of isolated hepatocytes.

METHODS

Suspensions of isolated hepatocytes (80-90% viable as judged by trypan blue exclusion) were prepared from 4-6 week-old male Wistar rats fed *ad libitum* as previously described (9). The intracellular K^+ concentration of the freshly-isolated cells (130-150 meq/liter cell water) did not vary significantly over a 60 min period of incubation at 37°. Unless otherwise indicated, experiments were carried out at 37° in Krebs Ringer bicarbonate media containing 3% (W/V) bovine serum albumin (Fraction V) and 1 mg/ml bacitracin as inhibitor of glucagon inactivation (10), and gassed with 5% CO_2-95% O_2. Bacitracin did not influence the basal rate of amino acid uptake. Cells (0.5 to 1.0 x 10^6/ml) were incubated with or without hormone and other agents at the concentrations and for the times indicated prior to addition of a mixture of labeled (0.05-0.1 µCi/tube) and unlabeled amino acid. Incubations were terminated by adding 0.1 ml of incubation mixture to 1.0 ml of chilled buffer. Cells were isolated by centrifugation, washed once with 1.0 ml of buffer, and transferred into counting vials. Trapping and nonspecific uptake did not exceed 5% of total uptake (7).

Aqueous cell volume was determined by incubating hepatocytes with ^3H_2O and with [^{14}C] polyethyleneglycol for 10 or 30 min at 37°. Measured values of intracellular water (^{3}H space minus ^{14}C space) : 3.35 ± 0.2 µl/10^6 cells (n=3) were identical after 10 or 30 min incubation times and were not affected by removing sodium (replaced by choline). Cell number was determined by counting cell suspensions in a Nageotte chamber. Counting was repeated on cells after incubation under conditions similar to those of the experiments. [1-^{14}C] AIB and [1-^{14}C] cycloleucine were purchased from Amersham. The amino acid analog α-(Methylamino)-isobutyric acid (N-methyl-AIB) was a gift of H.N. Christensen (Ann Arbor, Michigan). Glucagon was pork monocomponent n° 9969 from Novo.

RESULTS

Glucagon stimulated total AIB uptake (Fig. 1). However, the nonsaturable,

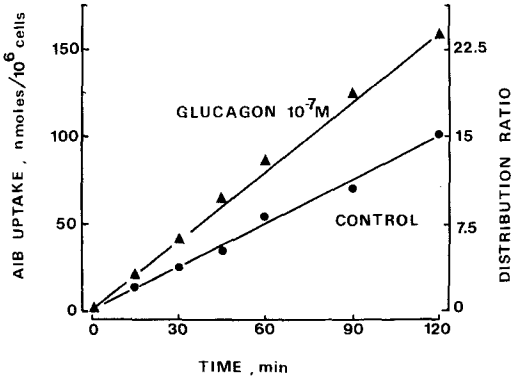

Figure 1. Effect of glucagon on the time course of AIB uptake in isolated hepatocytes. Cells were preincubated with or without glucagon for 30 min. At time 0 on figure , 2 mM AIB was added and AIB uptake was measured at the times indicated. The distribution ratio was calculated by dividing the intracellular concentration of AIB by the extracellular concentration. Each point is the mean of duplicate determinations.

TABLE I

Effect of Glucagon on Neutral Amino Acid Transport Systems in Isolated Hepatocytes.

Model Amino Acid Present During Transport Measurements	AIB Uptake, nmol/10^6cells/30 min	
	Without Glucagon	With Glucagon
AIB	3.33 ± 0.8	4.07 ± 0.05[a]
AIB + N-methyl-AIB	1.28 ± 0.05	1.23 ± 0.03[b]
	Cycloleucine Uptake, nmol/10^6cells/4min	
	Without Glucagon	With Glucagon
Cycloleucine	1.28 ± 0.04	1.38 ± 0.17[b]
Cycloleucine + AIB	1.58 ± 0.08	1.69 ± 0.18[b]

Cells were incubated without and with 10 nM glucagon for 30 min prior to the addition of amino acid. AIB (0.5 mM) uptake was measured as indicated in Methods, in the absence and presence of 20 mM N-methyl-AIB. Cycloleucine (0.2 mM) uptake was measured in sodium-free medium (sodium was replaced by choline), and in sodium-containing medium in the presence of 30 mM AIB (See Text for discussion of effects observed). Each value is the mean ± SEM of six determinations.

[a] Significantly different ($P < 0.001$) from basal (without glucagon)

[b] Not significantly different from basal (without glucagon).

sodium-insensitive component of AIB uptake (7) was not affected by the hormone (not shown). The effect of glucagon was strictly limited to the A system since the hormone was without effect on AIB uptake (at 0.5 mM) in the presence of 20 mM N-methyl-AIB (Table I). Under these conditions, we have observed that N-methyl-

AIB, which has been shown to react only with the A system in the Ehrlich cell (8), completely inhibits the A mediation of AIB transport in isolated hepatocytes (7). The N-methyl-AIB-insensitive part of AIB uptake, which was not affected by glucagon (Table I), is believed to represent transport through the ASC system (8).

The data do not suggest that glucagon may influence the L mediation (8) of transport. Thus glucagon was without significant effect on that part of cycloleucine uptake measured either in Na^+-free medium or in Na^+-containing medium in the presence of excess AIB (Table I), conditions under which cycloleucine is transported through the L system in isolated hepatocytes (7).

Glucagon increased the rate of AIB uptake, which was linear (with 2 mM AIB) over a 120 min period both in the absence and presence of the hormone (Fig. 1). Glucagon caused a similar increase (about 50%) at each time point. The concentrative nature of AIB transport is demonstrated by the high values of distribution ratio achieved (Fig. 1). Increasing the duration of exposure of cells to 10 nM hormone prior to the addition of AIB did not markedly enhance the magnitude of glucagon effect (Fig. 2). The relatively higher effect of glucagon observed at 120 min in Fig.2 has not been reproduced in two other experiments. In fact, the hormone effect could

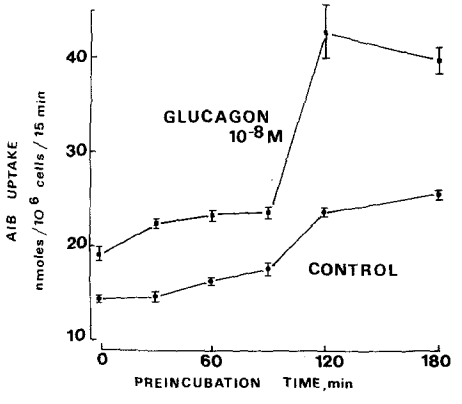

Figure 2. Effect of duration of cell exposure to glucagon on AIB uptake. Cells were preincubated in the presence or absence of glucagon for the times indicated prior to the addition of 2 mM AIB. Uptake was then measured over 15 min. Each point is the mean ± SEM of triplicate determinations.

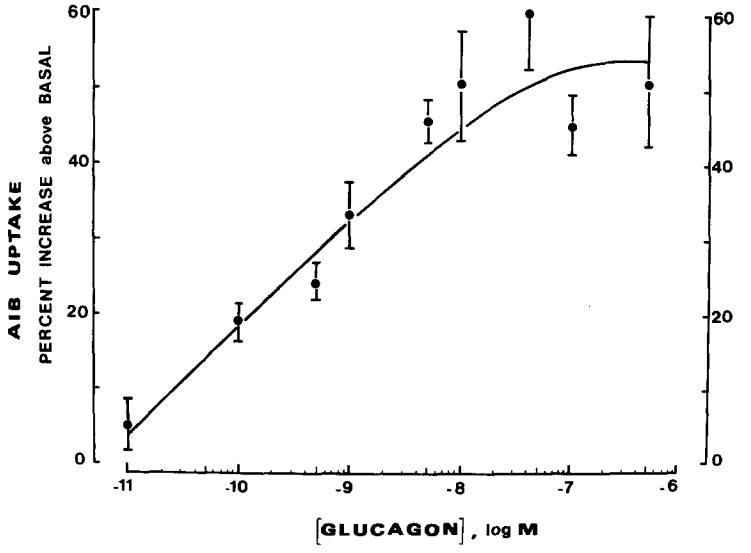

Figure 3. Dose response curve of glucagon stimulation of AIB uptake. Cells were preincubated with varying concentrations of glucagon for 30 min prior to the addition of 2 mM AIB. Uptake was then measured after 30 min. Each point is the mean ± SEM of four separate experiments. Within each experiment, four to six determinations were done for each concentration point.

be observed without preincubation of cells with glucagon, i.e., when cells were exposed to hormone and AIB together for only 15 min (Time 0 in Fig.2). The uptake of AIB (both basal and glucagon-stimulated) increased with the duration of cell preincubation (Fig. 2) as previously observed in liver slices (1).

Complete dose response curves were obtained with glucagon concentrations ranging from 0.01 nM to 100 nM (Fig. 3). Half-maximal stimulation was observed with glucagon at 0.40 ± 0.15 nM (mean ± SEM of four repeated experiments) ; maximal stimulation was achieved in the range of 10-100 nM hormone (Fig. 3). This is similar to the dose response relationship of glucagon-stimulated cyclic AMP accumulation in freshly-isolated hepatocytes (11). Dibutyryl cyclic AMP at 10 μM to 1mM stimulated AIB uptake to a degree similar to that observed with glucagon ; most of the stimulatory effect was already noted at 10 μM (Table II).

Preincubation for 60 min with cycloheximide blocked the glucagon stimulation of AIB transport (Table III). Cycloheximide also depressed slightly (but significantly) the basal rate of AIB uptake.

TABLE II

Effect of Dibutyryl Cyclic AMP (Dbc-AMP) on AIB Uptake in Isolated Hepatocytes

Dbc-AMP mM	0	0.01	0.10	1.00
AIB Uptake nmol/10^6cells/20 min	26.4 ± 0.5	35.7 ± 0.5[a]	38.8 ± 0.6[a]	39.8 ± 0.9[a]
Per Cent Increase		36	47	51

Cells were preincubated for 20 min in the absence and presence of varying concentrations of dbc-AMP. AIB (2 mM) was then added and uptake was measured after 20 min. Each value is the mean ± SEM of six determinations. In two other experiments, 1 mM dbc-AMP elicited 36% and 60% increase in AIB uptake.

[a] Significantly different ($P < 0.001$) from basal (0 dbc-AMP).

DISCUSSION

The present results have shown that freshly-prepared suspensions of hepatocytes transport AIB against a concentration gradient and that glucagon stimulates this active transport. In studies to be reported elsewhere (7), we have characterized neutral amino acid transport systems and demonstrated that, as in Ehrlich ascites tumor cells (8), the A, ASC and L systems are operative in freshly-isolated hepatocytes of adult rat. The present report provides, to our knowledge, the first direct demonstration that glucagon acts only upon one (the A system) of the specific mediating transport systems for neutral amino acids in the liver. Earlier studies *in vivo* had suggested that glucagon did not affect the L system (12).

The effect of glucagon on AIB transport was detected with hormone concentrations as low as 0.01-0.1 nM (35-350 pg/ml) and the concentration causing half-

TABLE III

Effect of Cycloheximide on Basal and Glucagon-Stimulated AIB Uptake

Glucagon	Cycloheximide mM	AIB Uptake nmol/10^6cells/30 min
No	None	17.2 ± 0.3 (a)
Yes	None	19.5 ± 0.3 (b)
No	0.1	14.5 ± 0.3 (c)
Yes	0.1	15.4 ± 0.5 (d)
No	1.0	15.9 ± 0.3 (c)
Yes	1.0	15.9 ± 0.4 (d)

Cells were incubated without and with cycloheximide for 60 min. Glucagon (10nM) was then added where indicated for a 30 min period prior to the addition of 2 mM AIB for another 30 min. Each value is the mean ± SEM of six determinations.

(b) Significantly different ($P < 0.005$) from (a)

(c) Significantly different ($P < 0.005$) from (a)

(d) Significantly different ($P < 0.005$) from (b)

(c) and (d) did not differ significantly.

maximal stimulation (0.4 nM, i.e., 1.4 ng/ml) was well within the physiological range of values observed in the pancreatico-duodenal vein *in vivo* (13). As in liver slices (1), dibutyryl cyclic AMP at low concentration (10 μM) was effective in stimulating AIB uptake.

There are at least two possible explanations for the increase in AIB uptake (both basal and glucagon-stimulated) observed in Fig. 2 : 1. The number of available carriers could increase following the release of intracellular amino acids. 2. A regulatory process, involving a repression-derepression mechanism by substrate molecules, i.e., amino acids, might be operative as reported for the A system

in chick embryo heart cells (14). Further work is in progress to test the latter possibility.

The effect of glucagon in stimulating AIB uptake was very rapid and, under the experimental conditions used in the present study, did not appear to markedly increase with the duration of cell exposure to hormone prior to the addition of the amino acid. This is slightly different from data reported by others in liver slices (1) where, although also observed without time lag, the glucagon effect increased as previous incubation of cells with the hormone was prolonged. We presently have no obvious explanation for this slight difference. It should be pointed out that variations in the magnitude of glucagon effect were observed, under similar experimental conditions, between individual preparations of cells. Whether such fluctuations depend on the isolation procedure itself or are related to the physiological state of the animal used as donor is subject to further investigation.

Our results differ from recent observations (5) in primary cultures of hepatocytes in which glucagon-stimulated AIB uptake was reported to occur after a 1-2 h time lag. In view of the rapidity of glucagon action and of the inhibitory effect of cycloheximide on both the basal and hormone-stimulated AIB uptake that we have observed, it is apparent that rapidly-turning over proteins are involved in AIB transport by the A system in freshly-isolated hepatocytes. In cultured liver cells, the half-life of these (or other) proteins may be longer, which could explain, partly at least, the difference observed. As reported by others in liver slices (2), our results are consistent with the rapid stimulation of liver amino acid transport observed *in vivo* following a high protein meal (15). This isolated cell system should prove useful in investigating the hormonal regulation of liver amino acid transport in various physiological and pathological states.

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