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Role of Microtubules in Insulin and Glucagon Stimulation of Amino Acid Transport in Isolated Rat Hepatocytes*

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The effects of the microtubule inhibitor, colchicine, on insulin or glucagon stimulation of α-amino[1-^{14}C]-isobutyric acid (AIB) transport were investigated in isolated hepatocytes from normal fed rats. Under all conditions tested, AIB uptake appeared to occur through two components of transport: a low affinity (K_m ~ 50 mM) component and a high affinity (K_m ~ 1 mM) component. Within 2 h of incubation, insulin and glucagon, at maximal concentrations, increase AIB (0.1 mM) uptake by 2- to 3-fold and 4- to 6-fold, respectively. Colchicine, at the low concentration of 5×10^{-7} M, slightly reduces basal AIB transport, decreases by 80% the stimulatory effect of insulin, and diminishes by 40% the stimulatory effect of either glucagon or dibutyryl cAMP. Kinetic analysis of AIB influx indicates that the drug inhibits the increase in V_{max} of a high affinity (K_m ~ 1 mM) component of transport stimulated by insulin or glucagon, without affecting the kinetic parameters of a low affinity component of transport (K_m ~ 50 mM). Various short term hormonal effects of insulin and glucagon (changes in glucose, urea, and lactate production) were found not to be modified by the drug.

Vinblastine elicits similar changes as colchicine on AIB uptake. Lumicolchicine, a colchicine analogue that does not bind to tubulin, has no effect. The concentration of colchicine (10^{-7} M) required for half-maximal inhibition of hormone-stimulated AIB transport is in the appropriate range for specific microtubule disruption.

These data suggest that microtubules are involved in the regulation of the insulin or glucagon stimulation of AIB transport in isolated rat hepatocytes.

Microtubules have been implicated in a variety of cellular functions such as maintenance of cell shape, chromosome displacement, saltatory movements, and secretory processes (see Ref. 1 for review). Moreover, recent evidences have suggested that these organelles play a role in the intracellular transmission of signals from cell surface receptors (2, 3) and the regulation of various hormonal responses (4-6). Colchicine, a drug perturbating microtubules (1), has been reported to potentiate hormone-stimulated cAMP production in leukocytes (4), lymphoma cells (5), and macrophages (7, 8). Furthermore, intact microtubules are required for the β-adrenergic- or cAMP-mediated increase in ornithine decarboxylase activity in a rat astrocytoma cell line (6), and *in vivo* experiments have shown that colchicine prevents the increase in amino acid transport in regenerating rat liver (9). Thus, the state of microtubule polymerization might be important for the regulation of the response of cells to various hormones or stimuli.

The present study attempts to elucidate whether intact microtubules are required for some of the hormonal responses of insulin and glucagon in isolated rat hepatocytes. Various short term hormonal effects (changes in glucose, urea, and lactate production) and a longer term effect, which is a protein synthesis-dependent process (AIB[1] transport), were measured. This last parameter was selected since AIB transport has been shown to be markedly stimulated by both insulin and glucagon within 2 h, due to the synthesis of a high affinity component of transport (10). The data indicate that microtubule disruption is associated with a marked diminution of the insulin- or glucagon-stimulated amino acid uptake, while the short term hormonal effects are unaffected.

MATERIALS AND METHODS

Chemicals—α-Amino[1-^{14}C]isobutyric acid (specific activity, 60 mCi/mmol) and L-[3,4 (*n*)-^{3}H]valine (specific activity, 15 Ci/mmol) were purchased from the Radiochemical Centre, Amersham (England). Collagenase and α-aminoisobutyric acid, were purchased from Sigma. All other organic and inorganic chemicals were purchased from E. Merck (Darmstadt, GFR), Fluka AG (Buchs, Switzerland), and Sigma and were of analytical grade. Lumicolchicine was prepared as described previously (11). Porcine insulin (10 times recrystallized) and glucagon (twice recrystallized) were from Novo Research Institute (Denmark). Bovine albumin fraction V from Sigma was defatted according to the method of Chen (12).

Animals—Ten- to 11-week-old male albino rats derived from a Wistar strain bred in these laboratories were used. They weighed between 220 and 270 g and were fed *ad libitum* with standard laboratory chow.

Preparation and Incubation of Isolated Rat Hepatocytes—Hepatocytes were prepared between 8 and 10 a.m. by collagenase digestion (13, 14). Following collagenase dissociation of the liver, cells were washed several times in a Krebs-Ringer bicarbonate buffer containing 2% (w/v) bovine serum albumin (Fraction V), as described previously (15). After washing, cells were preincubated for 20 min under an atmosphere of O_2:CO_2 (95:5, v/v), in a Krebs-Ringer bicarbonate buffer (pH 7.4, 37 °C) containing 3% (w/v) defatted bovine serum albumin, 150 mg/100 ml of glucose, and gentamycin (50 μg/ml). After preincubation, cells were centrifuged, resuspended in the same medium, and distributed as 2.5-ml aliquots (approximately 8 mg/ml wet weight) into 25-ml Erlenmeyer flasks. Incubations were then carried out in the presence or absence of drugs or hormones for varying lengths of time as indicated in figures. Colchicine or vinblastine was dissolved in isotonic NaCl and added to incubation vials as 100-fold concentrated solutions. Cell viability, estimated by the trypan blue

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[1] The abbreviations and trivial names used are: AIB, α-aminoisobutyric acid; BrcAMP, 8-bromo adenosine 3′:5′-monophosphoric acid.

exclusion test, was 90–95% after 150 min incubation and was not changed by drugs and hormones.

AIB Transport Studies—At the end of incubations with drugs and hormones, hepatocytes from each single 25-ml incubation vial were isolated by centrifugation (10 s at 500 × *g*), then resuspended in 1.35 ml of the same medium except that drugs and hormones were omitted. Cells were gased for 2 min and preincubated for 5 min in 10-ml Erlenmeyer flasks to let them recover from centrifugation. Transport experiments were then initiated by adding 150 μl of Krebs-Ringer bicarbonate buffer containing a mixture of [^{14}C]AIB (about 0.1 μCi) and unlabeled AIB to obtain the final desired concentration of amino acid. The experiment was stopped at appropriate times by a 30-s centrifugation of 750-μl samples through 400 μl of an oil mixture of dinonylphtalate:dibutylphtalate (1:3, v/v) at 8,000 × *g* in an Eppendorf 5412 microcentrifuge (16). Following centrifugation supernatants were discarded without removing oil. Tubes were then refilled on the top of the oil with cold saline and then saline plus oil were discarded. The insides of the tubes above the cell pellets were thereafter wiped dry with cotton swabs. Cell pellets were then resuspended by sonication in 1.6 ml of scintillation fluid (Lumagel, Lumac Systems A.G., Basel). Tubes were capped and transferred into counting vials for ^{14}C radioactivity determination. Blank tubes were identical to the experimental ones, except that the incubation was terminated immediately after addition of the labeled amino acid. Blanks never exceeded 10% of total radioactivity taken up at 0.1 mM [^{14}C]AIB.

For kinetic analysis of the Na^+-dependent AIB uptake, an experimental design described previously was used (10, 14). When a sodium-free medium was required, NaCl and $NaHCO_3$ were replaced by choline chloride and choline bicarbonate (choline/KRb medium). The Na^+-dependent AIB uptake was determined by substracting, at each substrate concentration, the values obtained in a Na^+-free medium from total velocities measured in the presence of Na^+ (14). At 0.1 mM AIB, a concentration of the amino acid primarily used in this study, the Na^+-independent component of transport was 11.1% of total transport (mean of 4 determinations) and was not changed by colchicine either alone or in combination with hormones. At higher substrate concentrations, the per cent of Na^+-independent component was greater but not altered by the drug. Results relating the Na^+-dependent velocity (v) to substrate concentration (S) were plotted as v against v/S (Eadie-Hofstee plot). When curvilinear plots were obtained, the assumption was made that two independent Michaelis-Menten components contributed to transport (10). Data were submitted to computer analysis, using a program kindly provided by Dr. Paul England, to obtain the fit of experimental points and the values of kinetic parameters. The mathematical method used involved a nonlinear least squares analysis of the theoretical equation of the hyperbola v (initial rate) against S (substrate concentration), describing a transport process where two independent carriers transport the same substrate: $v = [(V_{max_1} \times S)/(K_{m_1} + S)] + [(V_{max_2} \times S)/(K_{m_2} + S)]$.

Biochemical Determinations—After various incubation times, as described in the figure legends, incubations were stopped by transferring cell suspensions into conical centrifuge tubes placed in ice water. Tubes were centrifuged at 3,000 × *g* for 5 min at 4 °C. Pellets and supernatants thus obtained were used for measurements of cell wet weight and various metabolic indices. Aliquots of supernatants were used for glucose and urea determinations (17). Lactate was determined fluorimetrically on the neutralized perchlorate extract of the supernatant (17). The incorporation of [^{3}H]valine (5 mM, 0.66 μCi/ml) into total trichloroacetic acid-precipitable proteins (cellular + released) was carried out according to a technique previously described (18).

Expression of Results—All results are expressed per g or per mg wet weight of hepatocytes ± S.E., measured at the end of incubations. Although figures and tables often show results of one particular series of experiments, all experiments reported have been repeated 2–6 times, and experimental vials containing hepatocytes were run in duplicate to quadruplicate.

RESULTS

Effect of Colchicine on Insulin- or Glucagon-stimulated AIB Transport—Time course experiments (data not shown) have indicated that basal or insulin- and glucagon-stimulated AIB uptake, both in the presence or absence of colchicine, increased linearly with time for at least 8 min and with AIB concentrations ranging from 0.1 to 40 mM. Due to this, in all subsequent studies, the uptake of AIB was determined over a 5-min period to ensure that initial rates of uptake (influx) were measured.

As can be seen by Fig. 1, insulin and glucagon, both used at maximally stimulating concentrations, markedly enhance AIB transport. Colchicine alone slightly reduces basal uptake of AIB but considerably inhibits the insulin- or glucagon-stimulated AIB transport. The half-maximal inhibitory concentration of colchicine is approximately 10^{-7} M (Fig. 1). This concentration of the drug is in the range of the *in vitro* dissociation constant of colchicine binding to purified tubulin (19). Maximal inhibition is reached at about 2×10^{-7} M colchicine. Accordingly, all subsequent studies have been performed with the lowest concentration of the drug producing the maximal effect (5×10^{-7} M). Dose responses of insulin, glucagon, and dibutyryl cAMP stimulation of AIB transport indicate (data not shown) that, at submaximal insulin concentration (1–7 nM), colchicine totally suppresses the insulin effect while at higher concentrations of the hormone a 60–85% inhibitory effect by the drug is observed. In contrast, colchicine decreases but fails to completely abolish the glucagon stimulation of AIB uptake at submaximal concentraions of the hormone (0.5–5 nM). The inhibition at maximal glucagon concentrations (30–50% inhibition) is less than that observed with insulin. Dibutyryl cAMP has qualitatively the same effects as glucagon, both in the presence or absence of colchicine.

Kinetic Analysis of AIB Influx—The initial rate of AIB uptake (influx) was measured at various substrate concentrations, in the presence or absence of colchicine, insulin, and glucagon (Fig. 2). Plots of the initial velocity of the Na^+-

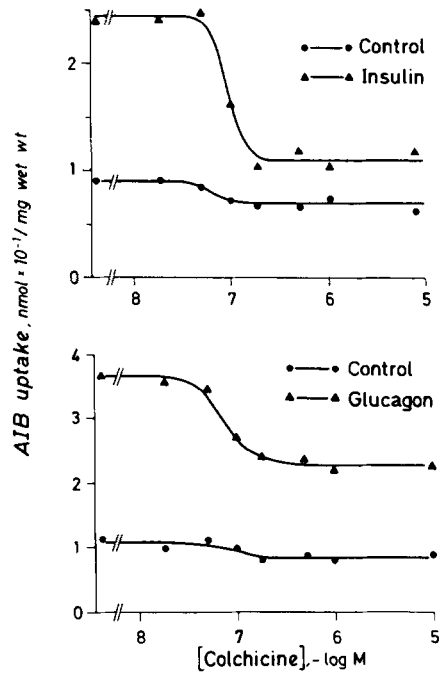

FIG. 1. **Effect of various concentrations of colchicine on the stimulation of AIB transport by insulin or glucagon.** Hepatocytes from fed rats were preincubated for 30 min at 37 °C with or without various concentrations of colchicine, in 2.5 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) that contained charcoal-treated bovine serum albumin (3.0 g/100 ml), glucose (150 mg/100 ml), and gentamycin (50 μg/ml). Insulin (35 nM) and glucagon (100 nM) were then added to incubation vials, and cells were further incubated for 2 h. After cell washing (see "Materials and Methods"), hepatocytes were resuspended in a fresh medium of the same composition containing neither drug nor hormones. Cells were then incubated in the presence of [^{14}C]AIB (0.1 mM, 0.07 μCi/ml) for 5 min. Each *point* is the mean of duplicate determinations.

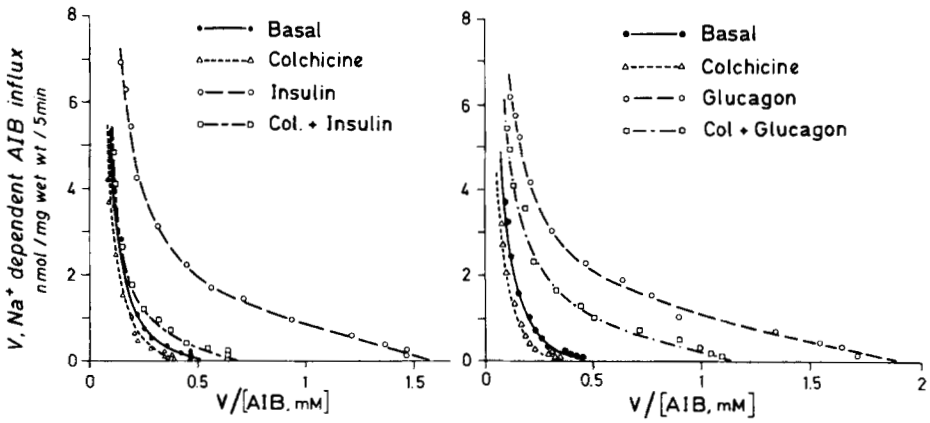

FIG. 2. **Effect of insulin and glucagon in the presence and absence of colchicine on the concentration dependence of AIB influx.** Hepatocytes were preincubated in the presence or absence of colchicine (5×10^{-7} M) for 30 min under the experimental conditions described in Fig. 1. Insulin (35 nM), glucagon (100 nM) or isotonic saline were then added to hepatocytes, and cells were further incubated for 2 h. Cells were then washed and incubated for 5 min in Na^+-containing medium and in Na^+-free medium at AIB concentrations ranging from 0.1 to 40 mM (0.2 μCi/ml). The Na^+-dependent velocity of transport was determined by substracting the Na^+-independent component from total transport at each AIB concentration. The data are presented in an Eadie-Hofstee plot where the Na^+-dependent transport (v) is plotted against v/[AIB] ratio. Each *point* is the mean of four separate experiments.

dependent transport (v) against (v/[AIB]) are curvilinear in all conditions tested (Fig. 2). Accordingly, all the different plots derived from experimental points were analyzed by computer, using a program that assumes two independent Michaelis-Menten components contributing to AIB transport in isolated rat hepatocytes (10, 20). Under basal conditions, the K_m of the low and high affinity components of transport are, respectively, 45 ± 4 mM and 0.9 ± 0.2 mM; the V_{max} of the low affinity component is 7.2 ± 0.3 nmol/mg wet weight·5 min. None of these parameters is changed by insulin or glucagon, either in the presence or the absence of colchicine. The values obtained for the V_{max} of the high affinity component (in nanomoles/mg wet weight·5 min) are as follows: basal, 0.29 ± 0.06; colchicine, 0.21 ± 0.02; insulin, 1.36 ± 0.13 ($p < 0.001$ *versus* basal, Student's t-test of paired data); insulin + colchicine, 0.61 ± 0.09 ($p < 0.01$ *versus* insulin alone); glucagon, 1.59 ± 0.3 ($p < 0.001$ *versus* basal); glucagon + colchicine, 1.00 ± 0.13 ($p < 0.05$ *versus* glucagon alone). Hence, insulin and glucagon increase by severalfold the V_{max} of the high affinity component of AIB transport, while colchicine markedly inhibits the increase in this V_{max} elicited by the hormone.

Additive Inhibition of Colchicine on the Combined Effects of Insulin and Glucagon on AIB Transport—When measured at 0.1 mM AIB, a concentration of substrate at which the contribution of the high affinity component to total transport is important, maximally stimulating concentration of insulin and glucagon exert additive effects on AIB uptake (Fig. 3). Interestingly, colchicine exerts an additive inhibition on the effect of insulin plus glucagon on AIB transport (Fig. 3).

Hormonal Stimulation of AIB Transport in the Presence of Colchicine, Lumicolchicine, and Vinblastine—Experiments were carried out to substantiate further the fact that colchicine inhibits the hormonal stimulation of amino acid uptake via microtubule perturbation. Vinblastine and colchicine, structurally different drugs that disrupt microtubules by apparently different mechanisms (1), both inhibit the insulin, glucagon, or BrcAMP stimulation of AIB transport (Table I). Furthermore, both drugs produce qualitatively similar changes: a marked inhibition of the insulin-stimulation of transport and less pronounced inhibition of glucagon-induced increase AIB uptake. Lumicolchicine, an analogue of colchicine that does not bind to tubulin nor disrupt microtubules (11), does not inhibit basal or hormone-stimulated AIB transport (Table I).

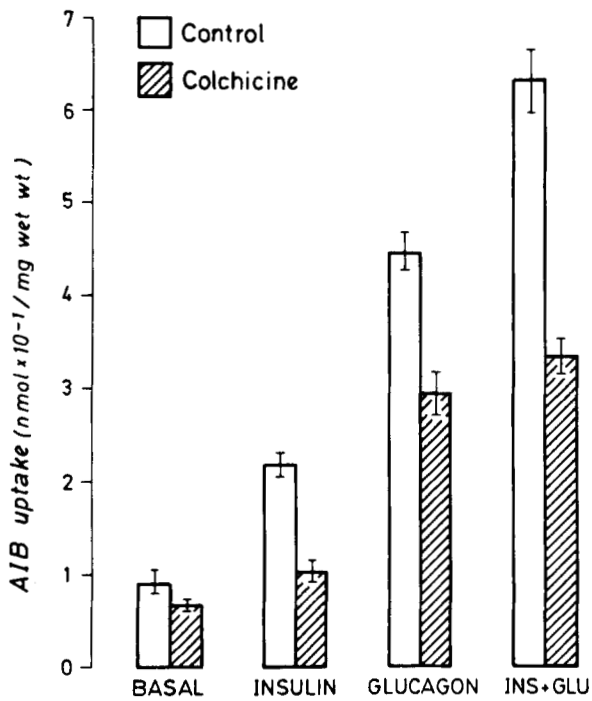

FIG. 3. **Effect of colchicine on the stimulation of AIB transport by insulin and glucagon.** Hepatocytes were incubated for 5 min with [^{14}C]AIB as described in Fig. 1. Colchicine, 5×10^{-7} M; insulin, 34 nM; glucagon, 100 nM. *INS*, insulin; *GLU*, glucagon. Each *bar* is the mean ± S.E. of triplicate determinations.

Protein Biosynthesis in the Presence of Colchicine, Insulin, and Glucagon—It has been demonstrated that insulin or glucagon stimulation of AIB transport requires protein synthesis (10). It was therefore necessary to determine whether the microtuble disrupting agent, colchicine, exerted its effects on the hormonal stimulation of AIB transport via an inhibition of protein synthesis. The incorporation of [^{3}H]valine (5 mM) into protein was measured. At this concentration of valine (an amino acid which is not degraded by the liver), the intracellular/extracellular specific radioactivity ratio approaches a value of 1 (21, 22). This method is therefore suitable to study protein synthesis in the presence of drugs or hormones which

TABLE I

Effect of colchicine, lumicolchicine, and vinblastine on the stimulation of AIB transport by insulin, glucagon, and BrcAMP

Hepatocytes from fed rats were incubated for 5 min with 0.1 mM [^{14}C]AIB under the experimental conditions described in Fig. 1. Results are means ± S.E. of triplicate determinations.

Addition	AIB influx
	nmol × 10^{-1}/mg wet wt
Experiment 1	
None	0.68 ± 0.06
Colchicine (5×10^{-7} M)	0.57 ± 0.06
Lumicolchicine (2×10^{-6} M)	0.66 ± 0.04
Insulin (35 nM)	2.01 ± 0.07
Insulin + colchicine	0.83 ± 0.08
Insulin + lumicolchicine	2.27 ± 0.09
Glucagon (100 nM)	3.72 ± 0.11
Glucagon + colchicine	1.98 ± 0.19
Glucagon + lumicolchicine	3.48 ± 0.08
Experiment 2	
None	0.94 ± 0.06
Vinblastine (5×10^{-7} M)	0.57 ± 0.04
Insulin (35 nM)	1.83 ± 0.13
Insulin + vinblastine	0.67 ± 0.06
Glucagon (100 nM)	4.06 ± 0.17
Glucagon + vinblastine	2.19 ± 0.19
BrcAMP (10^{-5} M)	4.35 ± 0.32
BrcAMP + vinblastine	2.28 ± 0.17

TABLE II

Protein biosynthesis in the presence of colchicine, insulin, and glucagon

Hepatocytes were incubated in the presence or absence of colchicine and hormones for 150 min with L-[3,4(*n*)-^{3}H]valine (5 mM, 0.66 μCi/ml). Results are means ± S.E. of triplicate determinations.

Addition	Valine incorporation into total protein
	μmol/g wet wt
None	2.49 ± 0.02
Colchicine (5×10^{-7} M)	2.35 ± 0.11
Insulin (35 nM)	2.88 ± 0.09
Insulin + colchicine	2.67 ± 0.15
Glucagon (100 nM)	2.14 ± 0.03
Glucagon + colchicine	2.07 ± 0.06

TABLE III

Lack of effect of colchicine on glucose, urea, and lactate production, both in the presence and absence of glucagon and insulin

Hepatocytes from fed rats were preincubated for 90 min with colchicine (5×10^{-7} M) under the experimental conditions described in Fig. 1. Hormones or isotonic saline were then added to incubation vials (time zero). Biochemical determinations were carried out after 40 min incubation and expressed as the differences from values at time zero. Negative values indicate net lactate uptake from incubation medium. Results are means ± S.E. of triplicate determinations.

Addition	Production		
	Glucose	Lactate	Urea
	μmol/g wet wt		
None	17.1 ± 5.1	8.66 ± 0.21	4.72 ± 0.19
Colchicine	15.9 ± 0.5	9.07 ± 0.42	4.40 ± 0.10
Glucagon (0.5 nM)	57.3 ± 3.6	−2.59 ± 0.35	6.56 ± 0.10
Glucagon + colchicine	50.2 ± 3.2	−0.72 ± 0.40	6.00 ± 0.21
Glucagon (5 nM)	70.7 ± 1.6	−9.46 ± 0.24	8.13 ± 0.42
Glucagon + colchicine	67.9 ± 1.3	−10.27 ± 0.24	7.43 ± 0.30
Insulin (35 nM)	3.9 ± 2.6	9.14 ± 0.21	4.36 ± 0.21
Insulin + colchicine	6.5 ± 3.0	9.07 ± 0.69	4.19 ± 0.03
Glucagon (0.5 nM) + insulin (35 nM)	39.2 ± 2.7	1.29 ± 1.13	5.55 ± 0.03
Glucagon + insulin + colchicine	30.5 ± 0.7	1.14 ± 0.64	5.08 ± 0.10

might change intracellular specific activity of an amino acid precursor. Under these conditions, the incorporation of [^{3}H]valine into protein is linear for at least 150 min (not shown). As illustrated by Table II, insulin slightly increases the incorporation of [^{3}H]valine into protein, while glucagon slightly inhibits this process. Colchicine, either alone or in combination with hormones, does not alter hepatic protein synthesis (Table II).

Short Term Hormonal Effects of Insulin and Glucagon in the Presence or Absence of Colchicine—As shown by Table III, the effects of glucagon and insulin, singly or combined, on glucose, lactate, and urea production are not modified by the presence of colchicine.

DISCUSSION

This study indicates that colchicine markedly inhibits insulin- or glucagon-stimulated AIB transport by isolated hepatocytes from fed rats. It is demonstrated that the drug specifically inhibits the increase in V_{max} of a high affinity hormone-stimulated component of transport ($K_m \sim 1$ mM), without affecting the kinetic parameters of a low affinity component of transport ($K_m \sim 50$ mM).

It is interesting to note that colchicine appears to inhibit AIB transport without regards to the agents (insulin, glucagon, dibutyryl cAMP, and α-adrenergic agonist (not shown)) used to stimulate amino acid uptake in hepatocyte. Several lines of evidence suggest that this colchicine effect is due to microtubule perturbation. First, the use of two structurally different microtubule disrupting agents (colchicine and vinblastine) elicit the same changes. Second, lumicolchicine, an isomer of colchicine that does not perturbate microtubules, has no effect on AIB uptake. Third, the concentration of colchicine (10^{-7} M) required for half-maximal inhibition of hormone-stimulated AIB uptake is similar to that reported for the inhibition of tubulin polymerization *in vitro* (23). This provides strong evidence that intact microtubules are required for insulin, glucagon, and cAMP stimulation of AIB transport in isolated rat hepatocytes.

The exact site of action of colchicine on this process is unknown. It is probably located at sites distal to hormone binding and cAMP metabolism. Indeed, dibutyryl cAMP enhancement of AIB uptake is inhibited to the same extent as glucagon, and the short term effects of glucagon and insulin are not affected by the drug (Table III). In addition, the binding of insulin to lymphocytes (24) and heart muscle (25) and the binding of growth hormone (24), epidermal growth factor (26), and β agonists (5) to culture cells are not altered by colchicine.

Although colchicine seems to interfere with the glucagon regulation of AIB transport at site(s) subsequent to the generation of cAMP, it does not appear that the inhibition of glucagon or insulin action on AIB uptake is due to a general effect on total protein synthesis. Indeed, the hormonal stimulation of AIB transport requires protein synthesis (10), but Table II indicates that the incorporation of [^{3}H]valine into total protein is unaffected by the drug. However, this observation does not preclude the possibility that colchicine inhibits the stimulation of AIB transport by specific effects upon protein synthesis or degradation, for example, the synthesis of a high affinity component of transport.

Although the present data do not allow us to locate exactly the site(s) of action of colchicine, two possibilities should be cited. 1) Microtubules might play a role in the transfer of hormonal information from the plasma membrane to intracellular sites concerned with the synthesis of a high affinity component of transport. This would be consistent with other reports (2, 3, 27) indicating that microtubules may convey

growth regulatory informations within the cell. 2) As colchicine inhibits the intracellular translocation and secretory process of protein in different cell types, including the liver (1, 18, 28), one might envisage that the intracellular transport of a high affinity component of transport to the plasma membrane is inhibited by the drug. Further studies are required to evaluate these possibilities which, however, are not exclusive.

Colchicine exerts an additive inhibition on the combined effects of insulin plus glucagon on AIB transport (Fig. 3). This indicates that the colchicine sensitive "sites" in the insulin or glucagon stimulation of AIB uptake are different.

In conclusion, microtubule integrity appears to be required for the hormonal stimulation of AIB transport in isolated rat hepatocytes. In particular, the data demonstrate that colchicine inhibits the increase of a high affinity component of transport ($K_m \sim 1$ mM) stimulated by insulin or glucagon. In contrast, the short term effects of these hormones do not appear to involve a colchicine-dependent step.

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