Biochimica et Biophysica Acta, 687 (1982) 35–41
Elsevier Biomedical Press

BBA 71097

PLASMA MEMBRANE VESICLES FROM ISOLATED HEPATOCYTES RETAIN THE INCREASE OF AMINO ACID TRANSPORT INDUCED BY DIBUTYRYL CYCLIC AMP IN INTACT CELLS

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(Received August 20th, 1981)

Key words *Amino acid transport, Plasma membrane vesicle, cyclic AMP, (Hepatocyte)*

We have investigated the effect of cyclic AMP on hepatic amino acid transport by measuring the uptake of L-alanine in plasma membrane vesicles purified from hepatocytes incubated without or with dibutyryl cyclic AMP. The application of an Na^+ gradient to vesicles from hepatocytes exposed to dibutyryl cyclic AMP, compared to control, resulted in a greater transient accumulation of L-alanine, whereas in the presence of a K^+ gradient a similar slow equilibration of L-alanine was observed. Kinetic analysis of L-alanine influx revealed that the increased uptake resulted from an increased capacity (V_{max}) with no change in the affinity (K_m) of the carriers for L-alanine. These results strongly suggest that dibutyryl cyclic AMP induces stable changes at the membrane level probably by increasing the number of amino acid carrier molecules.

Introduction

Previous work from this laboratory has shown that glucagon stimulates amino acid transport in freshly isolated hepatocytes by inducing the emergence of a high affinity transport component with the properties of a pure 'A' (alanine preferring) transport system [1]. This effect, which is fully mimicked by cyclic AMP [1], presumably represents an important regulatory step in liver metabolism and growth since fasting [2] and partial hepatectomy [3], both characterized by high levels of circulating glucagon, are also accompanied by the emergence of a high affinity amino acid transport component.

The physiological implications of these observations are, however, hampered by the fact that amino acid transport in isolated hepatocytes has been investigated using non-metabolisable synthetic analogues. Since the uptake of alanine and α-aminoisobutyric acid (a non-metabolisable analogue of alanine) have been reported to differ both quantitatively and qualitatively in freshly isolated hepatocytes [4], we have decided to investigate whether metabolically inactive membrane vesicles, prepared from isolated hepatocytes, retain amino acid transport changes after cell exposure to dibutyryl cyclic AMP.

Materials and Methods

Materials. L-[2,3-^{3}H]Alanine was purchased from the Radiochemical Centre (Amersham, U.K.) and Na^{125}I from the Commissariat à l'Energie Atomique (Saclay, France). Unlabelled L-alanine was from Merck (Darmstadt, F.R.G.). Other unlabelled amino acids, bovine serum albumin (fraction V), *p*-nitrophenyl phosphate, N^6,O_2-dibutyryl adenosine 3′:5′ cyclic monophosphoric acid were from Sigma Chemical Co. (St. Louis, MO). Cytochrome *c* (horse heart) was from Boehringer (Mannheim, F.R.G.). Porcine monocomponent insulin and porcine glucagon were generously supplied by

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the Novo Research Institute (Copenhagen, Denmark) and Novo (France). Other chemicals were of the best commercial grade available.

Preparation of isolated hepatocytes and incubation procedure. Male Wistar rats (140–150 g) were used throughout this study. Hepatocytes were isolated by collagenase dissociation of the liver as previously described [1,5]. The proportion of parenchymal cells (i.e., hepatocytes) in the purified cell suspension exceeded 95% [5]. Cell viability estimated by cell membrane refractoriness under phase contrast microscopy [1,5], ranged routinely between 90 and 95%.

Hepatocytes were resuspended (final concentration: $1.5 \cdot 10^6$ cells/ml) in a Krebs-Ringer bicarbonate buffer (pH 7.4) containing 10 mg/ml bovine serum albumin (fraction V), 0.8 mg/ml bacitracin and 50 μg/ml gentamycin and gassed with a mixture of 5% CO_2/95% O_2. Cells were incubated for 2 h at 37°C, with intermittent shaking, in the absence or presence of 0.1 mM dibutyryl cyclic AMP, in culture flasks (Falcon 75, 12 ml of cell suspension per flask). At the end of the incubation, the effect of dibutyryl cyclic AMP on amino acid transport was tested on a sample of each cell suspension by measuring the influx of 0.1 mM α-amino[^{14}C]isobutyric acid as previously described [1]. In all experiments α-aminoisobutyric acid influx was increased 3- to 5-fold when hepatocytes were incubated in the presence of dibutyryl cyclic AMP.

Preparation of plasma membrane vesicles. Hepatocytes were collected by centrifugation ($1000 \times g$, 2 min) either immediately after cell isolation or after a 2-h incubation without or with dibutyryl cyclic AMP; cells were then washed once in a buffer containing 250 mM sucrose, 0.2 mM $CaCl_2$, 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), equilibrated at pH 7.5 with KOH (homogenization buffer), and centrifuged again ($1000 \times g$, 2 min). The cell pellet was weighed, resuspended in 5 vol. of homogenization buffer, frozen in liquid nitrogen and stored overnight at −80°C before membrane vesicle preparation.

Just prior to preparing membrane vesicles, the frozen cell suspension was rapidly thawed at 40°C and diluted twice in the homogenization buffer Cells were disrupted in a Dounce homogenizer at 4°C (20–30 strokes) using a tightly fitted pestle. The plasma membranes were purified according to Van Amelsvoort et al. [6] except that the discontinuous density gradient was prepared from 13.5 ml of 46.5% (w/v) sucrose and 13.5 ml of 21.5% (w/v) sucrose. Purified plasma membrane vesicles were used either immediately after preparation or after one night at 4°C.

Enzyme assays. The K^+-dependent *p*-nitrophenylphosphatase activity of the (Na^+-K^+)-ATPase (EC 3.6.1.3) was determined as described by Gache et al. [7]. Glucose-6-phosphatase (EC 3.1.3.9) activity was measured by adding 0.1 ml of diluted homogenate or 0.1 ml of diluted vesicle suspension to 0.1 ml of glucose 6-phosphate (4 mM) solubilized in citrate buffer (0.1 M sodium citrate, 5 mM $MgCl_2$ (pH 6.5)). The mixture was incubated for 30 min at 37°C. The reaction was stopped with 1 ml of 10% trichloroacetic acid. After centrifugation for 10 min at $1000 \times g$ the liberated orthophosphate was assayed in the supernatant by the method of Ames [8]. The cytochrome *c* oxidase (EC 1.9.3.1) activity was assayed according to Cooperstein and Lazarow [9]. RNA was determined as described by Fleck and Begg [10] and proteins by the method of Lowry et al. [11].

Insulin and glucagon binding. Insulin and glucagon were iodinated to specific activities of 200–250 μCi/μg and 90–110 μCi/μg, respectively, as previously described [12]. Binding assays were initiated by adding 25 μl of homogenate or vesicle suspension (final protein concentration: 1.25 mg/ml) to plastic conical microfuge tubes (Eppendorf, 1.5 ml capacity) containing 155 μl of ^{125}I-labelled insulin (final concentration: 0.3 ng/ml) or ^{125}I-labelled glucagon (final concentration: 0.7 ng/ml) and 20 μl of either hormone-free buffer or buffer containing 10^{-6} M unlabelled insulin or glucagon. Both the labelled and unlabelled hormones were diluted in Krebs-Ringer bicarbonate buffer containing bovine serum albumin, gentamycin and bacitracin as indicated above, plus Trasylol at a final concentration of 1000 kI.U./ml. After 90 min at 20°C (condition under which both insulin and glucagon binding reached a steady state, data not shown) the assays were terminated by adding 1.0 ml of chilled saline to each tube and collecting membranes by centri-

fugation for 5 min at 8000 × *g* in a Beckman microfuge. Pellets were washed (without resuspension) with 1,0 ml of chilled buffer, centrifuged again, and counted for ^{125}I radioactivity in a gamma spectrometer at 60% efficiency.

Transport assays. Transport assays were carried out as previously described by Van Amelsvoort et al. [6] except that separate incubations were performed for each experimental condition, by adding 10 μl of membrane suspension to 10 μl of incubation mixture giving the following final concentrations: 3.5–5 mg of protein per ml, 250 mM sucrose, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$ and 10 mM Hepes-KOH (pH 7.5). L-[2,3-^{3}H]Alanine was present at a constant activity of 1 μCi per assay. Other additions are given in the legends to figures and tables. Alanine uptake was terminated by adding 1.5 ml of ice-cold buffer containing 100 mM NaCl, 250 mM sucrose, 0.2 mM $CaCl_2$ and 10 mM Hepes-KOH (pH 7.5). The diluted membranes were immediately filtered through a Millipore filter (HAWP, pore size 0.45 μm). The filter was washed twice with ice-cold dilution buffer. Filters were digested in 5 ml of Unisolve (Koch-Light Laboratories) and radioactivity was measured in a liquid scintillation spectrometer (Packard Tricarb).

Results

Purification of plasma membrane vesicles

Analysis of marker enzyme activities and hormone binding properties of the plasma membrane vesicle fraction obtained in three separate experiments is shown in Table I. The specific activity of the plasma membrane marker enzyme K^+-dependent *p*-nitrophenylphosphatase (ouabain-sensitive) [7] and the specific binding capacity of plasma membrane for insulin and glucagon were increased 12–14-fold relative to those found in the homogenate, with a mean recovery of approx. 55%. The specific activities of glucose-6-phosphatase (a marker enzyme of the endoplasmic reticulum) and of cytochrome *c* oxidase (a marker enzyme of mitochondria) were increased 2.5- and 1.5-fold, respectively, whereas the RNA content (a marker of the rough endoplasmic reticulum) remained unchanged. These results indicate that the purification procedure used in this study allows to prepare from isolated rat hepatocytes, large amounts (55% recovery) of purified (14-fold) plasma membranes, contaminated to a small extent by smooth endoplasmic reticulum and mitochondria.

Time course of L-alanine uptake

Freshly isolated rat hepatocytes were incubated for 2 h at 37°C, then frozen; membrane vesicles were prepared as indicated in Materials and Methods and the ability of the plasma membrane vesicles to transport amino acids was tested with L-[2,3-^{3}H]alanine. Fig. 1 shows the time course of L--alanine uptake in membrane vesicles. In the presence of an Na^+ gradient, a transient accumulation of L-alanine was observed. This overshoot

TABLE I

ENZYME SPECIFIC ACTIVITIES, INSULIN AND GLUCAGON BINDING CAPACITIES, AND RNA CONTENT OF HOMOGENATE AND PLASMA-MEMBRANE FRACTION FROM ISOLATED HEPATOCYTES

Specific activities are expressed as μmol of substrate transformed per h and per mg protein Insulin and glucagon specific-binding are expressed as fmol of hormone bound per mg protein (concentration of ^{125}I-labelled insulin and ^{125}I-labelled glucagon in binding assays were 50 pM and 200 pM, respectively). RNA content is expressed in μg per mg protein The protein content of the plasma membrane fraction represented 4 0±0 6% of the homogenate protein content Results are means±S E of three separate experiments

Parameters	Homogenate	Plasma membranes	Mean relative specific activity	Mean recovery (%)
Ouabain-sensitive K^+-dependent nitrophenylphosphatase	0.87±0 05	12 17± 0 97	14	55
Insulin binding	0 90±0 20	10 87± 1 16	12	53
Glucagon binding	9 58±2 05	136 57±31 33	14	59
Glucose-6-phosphatase	1.96±0 08	4 76± 0.36	2 5	9
Cytochrome *c* oxidase	28.64±5 24	40 96±10.82	1 5	6
RNA	30 93±3 13	29 80± 0 88	1	4

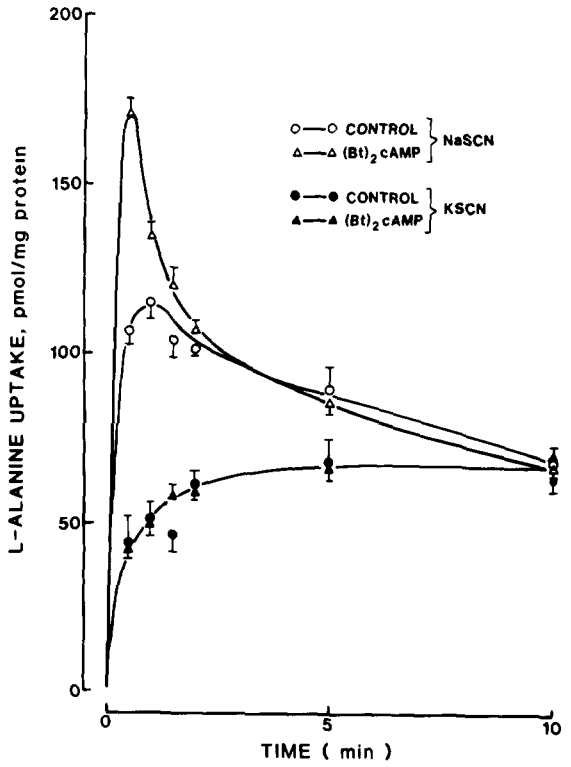

Fig 1 Time course of L-alanine uptake by plasma membrane vesicles prepared from hepatocytes incubated with or without dibutyryl cyclic AMP Plasma membrane vesicles were incubated at 25°C with 0 1 mM L-[2,3-^{3}H]alanine and 60 mM NaSCN or 60 mM KSCN Transport assay was carried out as described under Materials and Methods The results are means ±S E of three separate experiments

was maximal after 1 min and represented a 1.7-fold accumulation compared to the equilibrium value obtained after 10 min of L-alanine uptake. In the absence of an Na^+ gradient (KSCN replacing NaSCN) no accumulation could be observed over the equilibrium level. These results are comparable to those reported by Sips et al. in membrane vesicles prepared from intact rat liver [13] or isolated rat hepatocytes [14].

When plasma membrane vesicles were purified from hepatocytes which were exposed for 2 h to 0.1 mM dibutyryl cyclic AMP, a marked increase in the overshoot level of L-alanine uptake was observed in the presence of an Na^+ gradient (Fig. 1). In contrast, L-alanine uptake measured in a K^+ medium was not different in vesicles prepared from cells incubated in the absence or presence of dibutyryl cyclic AMP and reached an equilibrium level identical to that obtained in the presence of Na^+ in both types of vesicles. These results indicate that the increase of alanine uptake observed in vesicles from hepatocytes exposed to dibutyryl cyclic AMP is due to a specific increase in an Na^+-dependent transport process and not to an increase in the membrane permeability (passive diffusion), or to a difference in the intravesicular space accessible to alanine.

Kinetic analysis of L-alanine influx

To determine whether the change in L-alanine uptake observed in vesicles prepared from hepatocytes exposed to dibutyryl cyclic AMP resulted from an increased capacity of an Na^+-dependent amino acid transport system or from an increased affinity of the carrier for the substrate or from both, the initial velocity of L-alanine uptake (i.e. the uptake measured over a 10 s period) was measured at concentrations varying from 0.1 to 20 mM (Fig. 2) in the presence of an Na^+ gradient. The diffusion part of L-alanine uptake was determined graphically by plotting the initial velocity measured in the presence of NaSCN, against the L-alanine concentration in the medium (Fig. 2, left panel). This plot became linear for L-alanine concentrations above 10 mM. This linear component of uptake (i.e. the non-saturable component) was assumed to represent the diffusion component of L-alanine uptake. A linear regression analysis was performed to determine the slope of this linear part of the plot and the amount of L-alanine taken up by diffusion was calculated as the product of this slope by the extravesicular concentration of L-alanine. When the diffusion component was subtracted from total uptake, a saturable L-alanine transport was found in vesicles from control cells or from cells exposed to dibutyryl cyclic AMP (Fig. 2, middle panel).

The relationship between the initial rate (influx) of the Na^+-dependent transport (v) and the substrate (L-alanine) concentration was analysed according to the graphical method of Woolf-Augustinsson-Hofstee, where v is plotted against v/[Ala] (Fig. 2, right panel). A linear plot was obtained for both types of vesicles indicating that in each situation L-alanine is taken up by a single

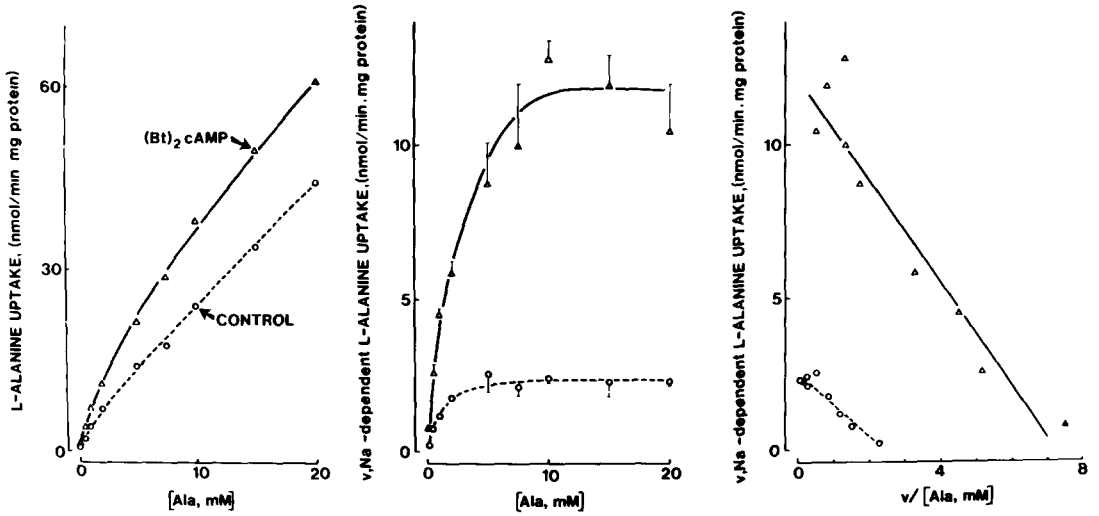

Fig 2 Concentration dependence of L-alanine uptake by plasma membrane vesicles prepared from hepatocytes incubated with or without dibutyryl cyclic AMP Plasma membrane vesicles were incubated at 25°C for 10 s with L-alanine concentrations ranging from 0 1 to 20 mM Left panel. L-alanine uptake measured in the presence of 60 mM NaSCN Middle panel· the Na^+-dependent part of transport was determined as indicated under Results Right panel the data relative to the Na^+-dependent transport were plotted according to Woolf-Augustinsson-Hofstee Results are means ± S E of three separate experiments

saturable component of transport. Moreover, the data in Fig. 2 (right panel) clearly indicate that the increase in L-alanine uptake observed in vesicles from hepatocytes exposed to dibutyryl cyclic AMP was totally accounted for by a 4–5-fold increase in V_{max} (2.6 ± 0.2 and 11.6 ± 0.9 nmol/min per mg protein; means ± S.E. of three separate experiments); the K_m was not significantly affected (1.1 and 1.5 mM).

TABLE II

AMINO ACID SPECIFICITY OF Na^+-DEPENDENT L-ALANINE UPTAKE

Plasma membrane vesicles were incubated with 0 1 mM L-[2,3-^{3}H]alanine, 60 mM NaSCN or KSCN and 5 mM of unlabelled amino acids Transport assays were carried out over 30-s periods Results represent the Na^+-dependent uptake of L-alanine transport (uptake measured in the presence of sodium minus uptake measured in the presence of potassium) and are the means ± S E of three separate experiments Statistical significance was assessed using Student's *t*-test for paired comparisons (no addition vs addition of unlabelled amino acid) All differences were significant ($P<0$ 05) except for D-alanine

Unlabelled amino acid added	L-Alanine uptake (pmol/30 s per mg protein)			
	Control vesicles		Vesicles from dibutyryl cyclic AMP-treated hepatocytes	
	Expt	%	Expt	%
No addition	57 ± 1	100	103 ± 3	100
L-Alanine	18 ± 2	32	28 ± 4	27
D-Alanine	49 ± 4	86	103 ± 10	100
L-Serine	22 ± 3	39	32 ± 3	31
α-Aminoisobutyric acid	46 ± 3	81	63 ± 8	61
α-(Methylamino)isobutyric acid	35 ± 4	61	48 ± 8	47

Amino acid specificity of Na^+-dependent L-alanine uptake

In a previous study from this laboratory [15] it has been shown that freshly isolated rat hepatocytes take up neutral amino acids through the major transport systems described in eukaryotic cells [16], namely systems A (alanine preferring), ASC (alanine, serine, cysteine preferring) and L (leucine preferring). A recent study indicated that another system (N) was also operative in the liver [17]. However, despite a few exceptions [17], only system A has been shown to undergo hormonal or non-hormonal regulation [18]. To characterize the specificity of the system(s) involved in the increase of amino acid transport induced by dibutyryl cyclic AMP, the uptake of 0.1 mM L-[2,3-^{3}H]alanine was measured in the absence or presence of an excess of various unlabelled amino acids. It should be pointed out that no saturable Na^+-independent uptake of L-alanine (measured in KSCN medium) could be demonstrated in the vesicles from control and dibutyryl cyclic AMP-treated hepatocytes, suggesting that system L did not contribute to alanine uptake. The Na^+-dependent L-alanine uptake was inhibited to a greater extent by amino acids sharing the 'A' mediation (i.e. alanine, serine, α-aminoisobutyric acid, α-(methylamino)isobutyric acid) in vesicles from hepatocytes exposed to dibutyryl cyclic AMP than in vesicles from control hepatocytes (Table II). The D stereoisomer of alanine did not significantly alter L-alanine uptake.

Discussion

In intact isolated hepatocytes glucagon and dibutyryl cyclic AMP increase the V_{max} of a high affinity amino acid transport component [1]. This increase in V_{max} could represent either changes in the number or mobility of the carrier molecules and/or changes in the driving force acting on the system. Glucagon and dibutyryl cyclic AMP have indeed been shown to enhance the transport activity of the (Na^+-K^+)-ATPase [19] and to induce an hyperpolarization [20] in hepatocytes. The possibility to evaluate amino acid transport in membrane vesicles in the presence of a controlled Na^+ gradient independently of the (Na^+-K^+)-ATPase activity led us to investigate the effect of hepatocyte exposure to dibutyryl cyclic AMP on L-alanine transport into purified plasma membrane vesicles. The results of the present study clearly indicate that vesicles prepared from hepatocytes exposed to dibutyryl cyclic AMP retain an increased amino acid transport capacity (V_{max}) in the presence of a controlled Na^+ gradient which is the driving force. Together with the observations that dibutyryl cyclic AMP stimulation of amino acid transport requires a 2–3-h hepatocyte exposure to the nucleotide and is totally inhibited by cycloheximide [1], the present results strongly support the hypothesis that dibutyryl cyclic AMP stimulates amino acid transport in hepatocytes by increasing the number (i.e. the synthesis) of carrier molecules.

Acknowledgements

We thank A. Kowalski for technical assistance, G. Visciano for illustration work and J. Duch for secretarial assistance. This study was supported by a grant 80.7.0187 from the Délégation Générale à la Recherche Scientifique et Technique.

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