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SUGAR TRANSPORT IN CHICK EMBRYO CARDIAC CELLS IN CULTURE

ANALYSIS BY COUNTERTRANSPORT, RELATIONSHIP TO PHOSPHORYLATION AND EFFECT OF GLUCOSE STARVATION

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Summary

Embryonic chick heart cells in culture transport 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose very rapidly. By direct measurements of uptake, it was not possible to estimate accurately transport rates, nor, with 2-deoxyglucose, to discriminate clearly between its transport and phosphorylation. In contrast, the technique of countertransport made it possible to determine precisely initial transport velocity and to make the following observations: (1) phosphorylation, and not transport, is rate-limiting in 2-deoxyglucose uptake; (2) hexose transport is stimulated 5-fold by removal of glucose from culture medium; and (3) this stimulation is followed by an increase in phosphorylation, but the effect is much less pronounced (2-fold stimulation only). In conclusion, the adaptative regulation of glucose transport described in many fibroblast cell lines exists also in cardiac cells.

Introduction

Heart cells from adult mammals and chick embryo use unesterified and esterified fatty acids as their major energy source [1]. We have recently studied fatty acid uptake in chick cardiac cells in culture [2—4]. Since glucose also represents an efficient nutrient, we decided to investigate hexose uptake and its regulation in these cells. Glucose starvation induces an increase in the activity of hexose transport in a number of cultured cells. This may involve a regulation of the synthesis and degradation of the carrier, as in chick fibroblasts [5,6], or simply an activation of pre-existing carrier molecules, as in Chinese hamster

fibroblasts [7]. The purpose of the present work was to study characteristics of sugar transport and to determine whether an adaptative regulation exists in cardiac cells, even though glucose is not their preferred substrate.

Materials and Methods

Cell culture. Chick cardiac cells were prepared from 10-day-old embryos as previously described [2], except that the medium used was Dulbecco's modified Eagle's medium (medium H 21 from Gibco) supplemented with 5% fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were initially plated at $2 \cdot 10^6$ cells/ml and the medium was changed after 24 h. For the glucose-starvation studies, cells were incubated in glucose-free medium supplemented with 5% dialysed fetal calf serum (Gibco).

Primary chick embryo fibroblasts were prepared from the body of the embryo by the method used for cardiac cells. Secondary cultures were obtained by trypsinization after 2 or 3 days of culture.

Monolayer cultures of cardiac cells are always contaminated by fibroblastic cells. Cellular heterogeneity was estimated by differential sensitivity of myoblasts and fibroblasts to 10^{-4} M veratridine [8]. After 24 h incubation with this alkaloid, secondary fibroblast cultures showed no significant alteration of cell density. In contrast, the cardiac preparation was dramatically lysed; less than 20—30% of the cells, judged to be fibroblasts, survived this treatment.

Hexose-uptake studies. Measurements were performed on confluent cells after 2—4 days of culture (0.5—1 mg protein per culture dish, 35 mm in diameter), 1 day after the medium (±glucose) was changed.

For direct measurement of hexose uptake, the medium was aspirated and the cells were washed twice with 2 ml Dulbecco's phosphate-buffered saline. At time zero, 1 ml of this medium, containing the labelled sugar, was added; the medium was aspirated at the indicated times and cells were washed four times with 2 ml of the same medium (4°C). Washing procedure took less than 15 s. Cells were then suspended in 0.1 M NaOH and radioactivity was counted by liquid scintillation spectrometry. An aliquot was held for protein determination [9]. (All results are expressed per mg protein).

For countertransport measurements, the cells were washed and incubated in phosphate-buffered saline containing 3-*O*-methylglucose (50 or 100 mM) for 30 min at the temperature of uptake (routinely 20°C). In some experiments, preloading of the cells was performed by adding 3-*O*-methylglucose directly to the culture medium, with identical results. After preloading, the medium was aspirated and the cells washed three times, in less than 10 s, with phosphate-buffered saline before the labelled medium was added. Uptake was then measured as indicated above. Data from experiments carried out under similar conditions indicated that experimental error in all uptake measurements did not exceed ± 7% (S.E., $n = 8$).

Determination of zero-time values with L-[^{3}H]glucose. Incorporation of L-[^{3}H]glucose was not significantly different between 0.25 and 60 min of incubation at 20°C. This indicates that, at this temperature, the rate of passive diffusion was too low to be detected above the background due to non-specific trapping of substrate in extracellular space. Therefore, L-glucose was used as a

marker of extracellular water space. This non-specific trapping was proportional to the external substrate concentration up to 20 mM; it was equal to approx. 0.06% of the total radioactivity present in the incubation medium. All uptake measurements were corrected for this value.

Intracellular water space. Intracellular water space was determined with tritiated water and poly([^{14}C]ethylene glycol) [2]. A value of 5.4 μl/mg protein was found.

Determination of phosphorylated 2-deoxyglucose. Phosphorylated sugar was precipitated as described by Kletzien and Perdue [10]. Free deoxyglucose was determined by counting an aliquot of the supernatant.

Results

Direct measurement of sugar uptake

Since D-glucose metabolism is very rapid and may lead to a significant excretion of lactate, we used two analogues to study the hexose transport: 3-*O*-methyl-D-glucose, which is not metabolized, and 2-deoxy-D-glucose which is only phosphorylated by the hexokinase, and trapped in the cell as phosphorylated sugar. Uptake measurements were performed at 15 or 20°C as indicated, in order to reduce the rate of transport.

A slight difference was observed in the uptake of 3-*O*-methylglucose by starved and glucose-fed cells (Fig. 1A). However, it was not possible to evaluate accurately either initial uptake velocity, since within 1 min the intracellular concentrations were 50% or more of the equilibrium value.

With 2-deoxyglucose, uptake was linear with both types of cell between 0.5 and 30 min (Fig. 1B). However, the curves did not extrapolate to the time-zero value. Moreover, when the phosphorylated and unphosphorylated sugars were estimated in the starved cells by the $Ba(OH)_2/ZnSO_4$ method, it was clear that free sugar accumulated in the cells in a manner similar to 3-*O*-methylglucose,

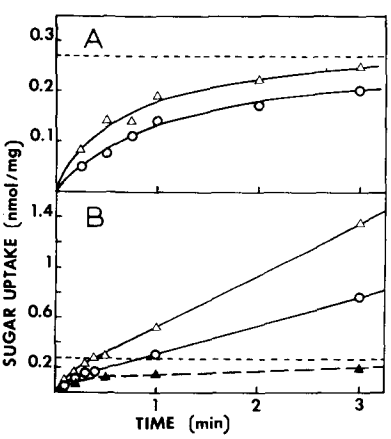

Fig. 1. Time course of uptake of 3-*O*-methyl[^{3}H]glucose and 2-deoxy[^{3}H]glucose. Cells were incubated for 20 h in medium with (○), or without (△) 25 mM glucose. Uptake of 3-*O*-methyl[^{3}H]glucose (A) or 2-deoxy[^{3}H]glucose (B) was measured at 20°C and at 50 μM substrate concentration. △———△ and ○———○ represent total uptake ▲- - - - -▲ represents unphosphorylated deoxyglucose in glucose-starved cells. The dashed line indicates the intracellular concentration equal to that in the extracellular medium.

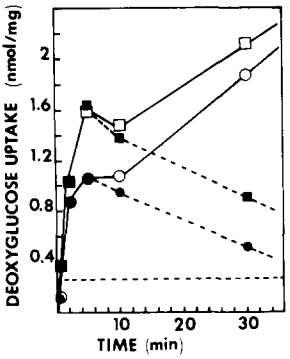

Fig. 2. Time course of 2-deoxy[^{3}H]glucose uptake in glucose-fed cells. 1 day after plating, the cells received fresh culture medium containing 30 mM glucose and were incubated for 20 h at 37°C. 1 h before the uptake measurement, the culture was divided in two, without medium change: half received 10 mM additional glucose (□ and ■), the other had no further addition (○ and ●). After washing, uptake of 2-deoxy[^{3}H]glucose (50 μM; 18000 dpm/nmol) was measured at 15°C. □———□ and ○———○, total uptake; ■- - - - -■ and ●- - - - -●, unphosphorylated sugar. The dashed line indicates the equilibrium value as in Fig. 1.

with the same approach to the intracellular-extracellular equilibrium level. Therefore, the linearity of uptake after 30 s more likely reflects phosphorylation of 2-deoxyglucose than its transport.

With glucose-fed cells, an interesting phenomenon could be occasionally observed when very young cultures (1—2 day old) were used. The uptake curve for 2-deoxyglucose showed an overshoot which could be correlated with a transient accumulation of unphosphorylated sugar far beyond the extracellular concentration (Fig. 2). This phenomenon, which could be enhanced by increasing the glucose concentration in the culture medium, was interpreted as a countertransport effect due to high internal free glucose levels. Labelled deoxyglucose was not phosphorylated during the first 5 min of uptake, possibly due to saturation of the hexokinase by unlabelled glucose and/or inhibition of the enzyme by glucose 6-phosphate [11]. These observations suggested that the technique of countertransport could be used to determine more accurately initial transport velocities.

Measurement of sugar uptake by countertransport

Cells were preloaded with a high concentration of unlabelled free sugar in order to saturate sites at the inner side of the membrane and thereby minimize export of the labelled substrate. Under these conditions, the uptake of labelled hexose remains a true unidirectional flux for a longer time than it does using direct measurements. This phenomenon has been described for numerous transport systems in a variety of cells [12]. As preloading sugar we chose the non-metabolized analogue, 3-*O*-methylglucose, rather than 2-deoxyglucose in order to avoid an increase in the intracellular hexose phosphate concentration, which could play a role in the regulation of sugar transport and phosphorylation [5].

When the cells were preloaded with 50 mM 3-*O*-methylglucose, the uptake of 50 μM 3-*O*-methyl[^{3}H]glucose was greatly enhanced above the equilibrium with the external medium (Fig. 3). It remained linear with time for about

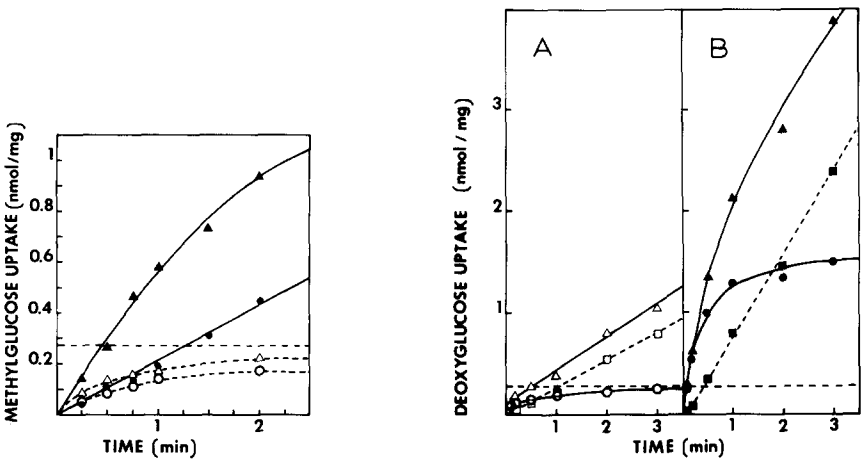

Fig. 3. 3-*O*-Methyl[^{3}H]glucose uptake in countertransport experiments. Cells were incubated for 20 h at 37°C either with (● and ○) or without (▲ and △) 25 mM glucose. Uptake of 3-*O*-methyl[^{3}H]glucose (50 μM) was measured at 20°C, either directly as in Fig. 1A (△------△ and ○------○), or after preloading with 50 mM 3-*O*-methylglucose (▲——▲ and ●——●). The horizontal dashed line refers to the equilibrium value. The initial transport rates, measured after preloading, were 0.22 and 0.60 nmol/min per mg for glucose fed and starved cells, respectively.

Fig. 4. Short-term effect of preloading on 2-deoxy[^{3}H]glucose uptake and phosphorylation in starved cells. After 20 h of glucose starvation, cells were either not preloaded (A) or preloaded (B) with 100 mM 3-*O*-methylglucose added directly into the culture medium (30 min, 37°C). Uptake of 2-deoxy[^{3}H]-glucose (50 μM) was measured at 20°C. △——△ and ▲——▲, total uptake; ○——○ and ●——●, unphosphorylated sugar; □------□ and ■------■, phosphorylated sugar (obtained by difference). The horizontal lines refer to the equilibrium value as in the previous figures.

1 min in starved cells, and for more than 2 min in cells grown in the presence of glucose. It must be pointed out that with the latter cells the same uptake was measured whether or not glucose (25 mM) was present in the preloading medium. Under these conditions, the transport rates could be correctly evaluated, and a 2.7-fold stimulation of 3-*O*-methylglucose uptake by removal of glucose could be demonstrated. Under the same preloading conditions, uptake of 2-deoxy[^{3}H]glucose was linear for at least 30 s in glucose-fed cells. With starved cells, preincubation with 100 mM 3-*O*-methylglucose was necessary to determine the initial uptake rate (Fig. 4). The initial rates were 0.55 and 2.75 nmol/min per mg protein for glucose-fed and starved cells, respectively. Phosphorylation rates were determined between 5 and 20 min, by the $Ba(OH)_2$/$ZnSO_4$ method, without preloading of the cells. These rates were 0.12 and 0.27 nmol/min per mg protein for glucose-fed and starved cells, respectively. Thus, transport of 2-deoxyglucose is faster than its phosphorylation both in fed and starved cells. Without preloading, the rate of transport of 2-deoxyglucose and its 5-fold stimulation by glucose removal are greatly underestimated. It is interesting to note that the phosphorylation rate was also increased when glucose-starved cells were preloaded with a high concentration of 3-*O*-methylglucose (Fig. 4B), due to the high level of free 2-deoxy[^{3}H]glucose in the cell (up to 280 μM for an external concentration of 50 μM). This indicates that the hexokinase has no affinity for 3-*O*-methylglucose [13]. With increased incubation time, the internal concentration of unphosphorylated 2-deoxyglucose

decreased, and there was a concomitant decrease in the phosphorylation rate, which became equal to that obtained with unpreloaded cells after about 15 min.

Kinetic parameters for 3-O-methylglucose and 2-deoxyglucose uptake

The initial rate of uptake of 3-*O*-methyl-D-[^{3}H]glucose was measured with increasing concentrations of substrate after cells were preloaded with 100 mM unlabelled 3-*O*-methylglucose (Fig. 5A). For starved cells, a K_m of approx. 10 mM and a V of approx. 200 nmol/min per mg were obtained from a Lineweaver-Burk plot. For glucose-fed cells the saturation was less pronounced and kinetic parameters could not be precisely determined. It is thus difficult to assess whether glucose removal affects V or K_m or both. At substrate concentrations up to 2 mM, the initial rate of transport of 3-*O*-methylglucose was approx. 5-fold stimulated by starvation (Fig. 5A).

When the same experiments were performed with 2-deoxy-D-[^{3}H]glucose, we observed a K_m of about 2 mM for both starved and fed cells, and a V about 5-fold higher in starved cells (Fig. 5B).

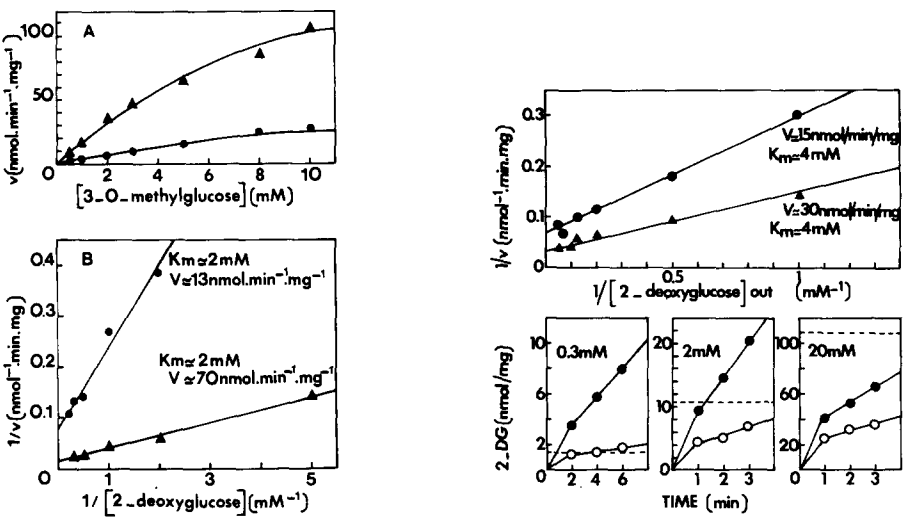

Fig. 5. Kinetics of 3-*O*-methyl[^{3}H]glucose and 2-deoxy[^{3}H]glucose transport in glucose-starved and fed cells. Cells were incubated for 20 h at 37°C either with (●), or without (▲), 25 mM glucose. After preloading with 100 mM 3-*O*-methylglucose, the effect of 3-*O*-methyl[^{3}H]glucose concentration (A) or 2-deoxy-[^{3}H]glucose concentration (B) on uptake was examined (20°C). Initial transport rates (v) were estimated by measuring incorporation after 0.1, 0.2 and 0.3 min. Over the range of concentrations tested, uptake remained linear during this time interval, and extrapolation to time zero gave a value identical to that obtained with L-[^{3}H]glucose. The increase in absolute extracellular concentration due to efflux of unlabelled 3-*O*-methylglucose was too low in 0.3 min to affect appreciably the apparent transport velocity. It could be estimated that the error in K_m was less than 10%, with no effect on the value of V.

Fig. 6. Kinetic analysis of the long-term incorporation of 2-deoxy[^{3}H]glucose in glucose-starved and fed cells. Cells were incubated for 20 h at 37°C in the presence (● and ○) or absence (▲) of 25 mM glucose. Uptake of varying concentrations of 2-deoxy[^{3}H]glucose was then examined (20°C). The v values indicated in the Lineweaver-Burk plot were calculated from the slope of the linear portion of the uptake curves between 2 and 6 min for concentrations up to 1 mM and 1 and 3 min for the higher concentrations (lower panel). ●———●, total uptake; ○———○, unphosphorylated sugar. The dashed line refers to the equilibration with the outer medium.

Analysis of the second phase of the uptake, which roughly reflects phosphorylation, indicated only a 2-fold increase in V. It must be emphasized that the K_m value of about 4 mM obtained for both types of cell does not exactly reflect the affinity of the hexokinase, since, with increasing external concentration of 2-deoxyglucose, the intracellular concentrations of free sugar became increasingly lower than the equilibration level (Fig. 6). Since no real steady state was attained, it was impossible to determine accurately the concentration of substrate available to the hexokinase and thus to measure its true K_m. However, this parameter could be evaluated to be at least 2-fold lower than that determined with the external concentration. Accordingly, the hexokinase could have nearly the same affinity as the transport system for deoxyglucose, a result similar to that obtained by Graff et al. [14] with Novikoff rat hepatoma cells.

Discussion

Hexose transport in cultured animal cells is generally measured by determining the rate of uptake of 2-deoxy-D-glucose. This method is valid, providing transport, and not phosphorylation, is rate-limiting in the uptake process. As underlined recently by Graff et al. [14], a rather complex relationship may exist in vivo between rates of transport and phosphorylation, and this relationship must be established for each cell system and each set of experimental conditions. In our system of confluent cardiac cells from chick embryo, it is clear that under all conditions, the rate of transport exceeds that of phosphorylation by several-fold. Accordingly, our data are more similar to those obtained by Graff et al. [14] with Novikoff rat hepatoma cells than to those of Kletzien and Perdue [5] with chick embryo fibroblasts.

A quasi steady-state concentration of free sugar is rapidly established in the cardiac cell. This internal pool, which levels off at a value below the K_m value for hexokinase, even at physiological concentrations of external glucose (Fig. 6), will therefore determine the rate of the phosphorylation step. Indeed, we found that (1) when the intracellular concentration of labelled substrate was artificially increased by countertransport, the phosphorylation rate was enhanced, and (2) when the intracellular concentration was decreased by cytochalasin B, phosphorylation decreased. Therefore, any modification of transport activity will be followed by a significant variation of the phosphorylation process and would be detected in measurements with 2-deoxyglucose, under all conditions which affect the intracellular free sugar concentration. However, the absolute transport rate would always be underestimated by measurement of long-term 2-deoxyglucose uptake. With our cells, it was necessary to use the technique of countertransport to assess accurately the rate of hexose transport, since it is much too rapid to be correctly measured either by the overall uptake of 2-deoxyglucose or by the transport of 3-*O*-methylglucose. Some reservations must be made concerning the use of this technique. Preloading might result in some increase in the catalytic efficiency of the carrier, which is usually designated as 'transactivation' or 'accelerative exchange diffusion'. This phenomenon did not seem to occur in our system, since we did observe the same initial slope for the uptake curves with and without preloading, whenever the transport process was not too rapid (e.g., Fig. 3 for glucose-fed cells). In any case,

even if some transactivation were to occur, the countertransport technique would allow measurement of the maximal rate of unidirectional flux. Therefore, it should permit a better comparison of the total activity of hexose transport in glucose-fed and starved cells.

Our data clearly show that the concentration of glucose in the culture medium can modulate hexose transport in chick cardiac cells. Moreover, although fatty acids are considered to be the preferred substrate of the heart muscle [1], inclusion of oleic acid, and even prolonged incubation of the cells with oleate and carnitine, did not alter hexose uptake. In this respect, the cultured cardiac cells do not seem to behave as the perfused adult rat heart muscle [15]. This discrepancy could not be accounted for by high contamination of the culture by fibroblastic cells. All of our studies were carried out on young cultures, in which the proportion of fibroblasts has been reported not to exceed 20—30% [16]. We confirmed this by comparing the lytic effect of veratridine on cardiac cells and on pure fibroblast secondary cultures. This alkaloïd is known to stimulate Na^+ influx in excitable cells, including embryonic cardiac cells in culture [8] and in this manner accounts for the selective lysis.

In studies with pure fibroblast cultures, we observed absolute values for transport rates (expressed per mg protein) and effects of glucose removal that were similar to those obtained with the cardiac cell preparations. Thus, it appears that fibroblast contamination alone cannot account for the transport phenomena observed in cultures of cardiac cells. Therefore, we conclude that cultured chick cardiac cells like fibroblasts have a regulation system for the hexose uptake, which responds to the external substrate concentration.

Two modes of regulation of hexose transport have been reported. One system, which has been described for chick fibroblasts, involves protein synthesis [6]. The other, described for Chinese hamster fibroblasts, involves a mechanism of activation [7]. In chick cardiac cells we observed a cycloheximide effect quite similar to that observed by Amos et al. [6]. Thus, the regulation of hexose transport in these cells is likely to involve primarily, as in chick fibroblasts, a variation in the number of active carrier molecules.

Does the external glucose concentration also regulate phosphorylation, or are the changes observed merely a consequence of altered transport activity? When total hexokinase activity was measured in vitro with cellular homogenates, the results were identical for glucose-fed and glucose-starved cells. The same result has been obtained with chick fibroblasts [5]. Therefore, the synthesis and turnover of the phosphorylating enzyme is obviously not regulated by the glucose concentration. However, the possibility cannot be ruled out that hexokinase activity could be modulated in vivo according to the concentration of some crucial metabolite(s) derived from glucose.

Acknowledgements

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