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Rapid stimulatory effect of insulin on binding of glycolytic enzymes to cytoskeleton of C-6 glial cells, and the antagonistic action of calmodulin inhibitors

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Insulin was shown in our previous experiments to induce an increase in binding of glycolytic enzymes to muscle cytoskeleton. We show here the same stimulatory effect of insulin in C-6 glial cells in culture. In these cells, like in muscle, a short time of incubation with insulin (1–10 min) induced an increase in cytoskeleton bound phosphofructokinase and aldolase. This stimulatory effect of insulin could be prevented by treatment with calmodulin antagonists trifluoperazine, thioridazine or CGS 9343 B (a potent and selective inhibitor of calmodulin activity), which strongly suggests that calmodulin is involved in this action of insulin. Our previous experiments have shown that growth factors and Ca^{2+} also induce a rapid, calmodulin-mediated stimulation of binding of glycolytic enzymes to cytoskeleton. The present and previous results suggest that the rapid binding of glycolytic enzymes to cytoskeleton, may be a general mechanism, in different cells, in signal transduction of insulin, growth factors and other Ca^{2+}-mobilizing hormones. The accelerated cytoskeletal glycolysis will supply local ATP, which is required for the rapid cytoskeletal-membrane rearrangements following the binding of hormone to its receptor.

Keywords: insulin; glycolytic enzymes; cytoskeleton; C-6 glial cells; calmodulin antagonists; phosphofructokinase

Introduction

Glycolytic enzymes are known to be regulated by allosteric regulators (see reviews Beitner, 1985, 1990, 1993), as well as by reversible binding to cytoskeleton (Arnold & Pette, 1968; for reviews see Clarke *et al.*, 1985; Beitner, 1993; Pagliaro, 1993). The latter mechanism has recently attracted much attention. It has been shown by many laboratories that glycolytic enzymes bind reversibly to cytoskeletal elements like actin, tubulin and microtubules (Arnold & Pette, 1968; Masters, 1984; Clarke *et al.*, 1985; Pagliaro & Taylor, 1988; Walsh *et al.*, 1989; Lilling & Beitner, 1990; Lilling *et al.*, 1991; Ovadi & Orosz, 1992; Beitner, 1993; Lehotzky *et al.*, 1993; Pagliaro, 1993). This binding affects the kinetic properties of the enzymes leading to their activation. The binding of glycolytic enzymes to cytoskeletal elements also provides a more efficient glycolytic flux since the enzymes form clusters that allow channeling of the substrates (Clarke *et al.*, 1985). This leads to the production of local ATP in the vicinity of the cytoskeleton, which is known to interact dynamically with plasma membrane upon membrane-induced events. The binding of glycolytic enzymes to cytoskeleton also affects cell structure (Clarke *et al.*, 1985).

Previous experiments in our laboratory have revealed that in muscle, insulin and growth factors, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), rapidly stimulate the binding of glycolytic enzymes to muscle cytoskeleton (Chen-Zion *et al.*, 1992a,b; Livnat *et al.*, 1993a, 1994). We also found that a physiological rise in intracellular Ca^{2+} exerted a similar increase in glycolytic enzyme binding (Chen-Zion *et al.*, 1993). The increase induced by all these factors could be prevented by calmodulin antagonists (Chen-Zion *et al.*, 1992b, 1993; Livnat *et al.*, 1993a, 1994), which strongly suggests that the binding of glycolytic enzymes to muscle cytoskeleton is mediated through Ca^{2+}-calmodulin complex.

Our experiments in muscle which have revealed the stimulatory action of insulin on cytoskeletal glycolysis (Chen-Zion *et al.*, 1992a,b), prompted us to investigate whether this mechanism is common to other tissues. In the present work we examined the effect of insulin on the binding of phosphofructokinase (PFK) and aldolase to cytoskeleton in C-6 glial cells in culture. Insulin was shown to stimulate protein synthesis is developing brain (Pal & Bessman, 1988). C-6 glial cells have been used extensively as a model system for studies of factors which play a role in the function of glial cells (Mangoura *et al.*, 1989). Moreover, these cells are capable of powerful glycolytic activity (Ercinska & Silver, 1987). Glucose transporter of glial cells was shown to be insulin dependent, while glucose transporter of neurons was insulin independent (Clarke *et al.*, 1984; Wei & Yeh, 1991). It was also reported that insulin increases glial glycogen levels which supports the neighboring neurons during glucose deprivation (Swanson & Choi, 1993). Since glial cells appear to be the main target cells for insulin action in the brain (Clarke *et al.*, 1984; Wei & Yeh, 1991; Swanson & Choi, 1993), we have chosen to use these cells to study insulin action on cytoskeletal glycolysis.

We show here, for the first time, that similar to its action in muscle, insulin increases the binding of PFK (the rate-limiting enzyme of glycolysis) and aldolase to cytoskeleton in glial cells. We also show that calmodulin antagonists prevent this effect of insulin.

Results

The effect of insulin on the binding of PFK and aldolase to cytoskeletal elements was studied in C-6 glial cells. Electron micrograph of negatively stained preparation shows that the 'bound fraction' of C-6 glial cells contains many cytoskeletal elements, which are similar by size to actin and microtubule filaments (Figure 1). Figure 2 shows the dose-response curves of the stimulatory effect of insulin on binding of PFK and aldolase to cytoskeleton in glial cells incubated in the presence of different concentrations of insulin. It can be seen that a significant effect was already observed with 0.27 mu ml^{-1} insulin, reaching a maximum stimulation at a concentration of 27 mu ml^{-1} insulin. Figure 3 shows the time-response curves of the effect of insulin on binding of glycolytic enzymes. It can be seen that the stimulatory effect of insulin on both enzymes was very rapid and transient. The increase in PFK and aldolase binding was already significant after 1 min of incubation with insulin. It reached a maximum between 3–5 min and thereafter declined.

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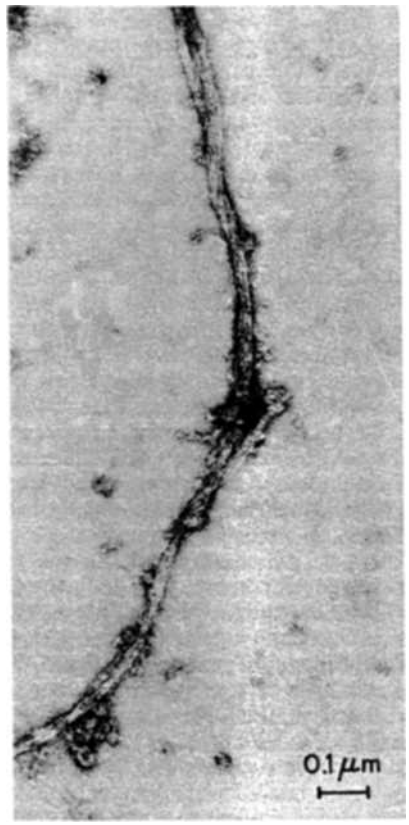

Figure 1 Electron micrograph of C-6 glial cells 'bound fraction'. 'Bound fraction' was separated and negatively stained as described under Materials and Methods (×70,000)

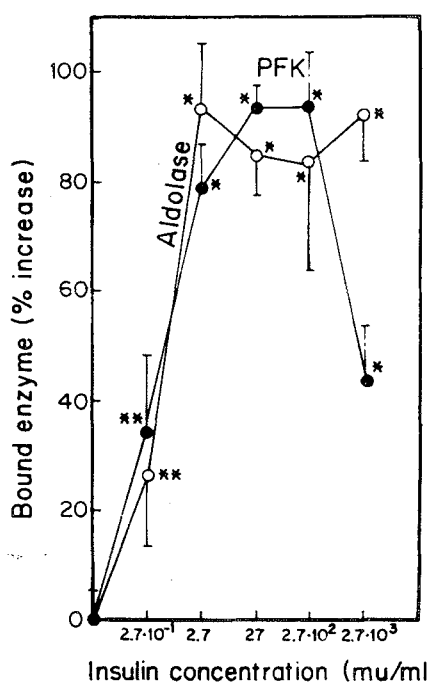

Figure 2 Dose-response curves of the effect of insulin on the binding of PFK and aldolase to cytoskeleton in C-6 glial cells. Cells were incubated for 5 min in absence and presence of different concentration of insulin. Each point is the mean ± SEM for 6–12 experiments. Activity of bound PFK and aldolase in the absence of insulin was 2.80 ± 0.35 and 8.05 ± 1.42 (mu/mg protein), respectively. $*P<0.005$, $**P<0.01$

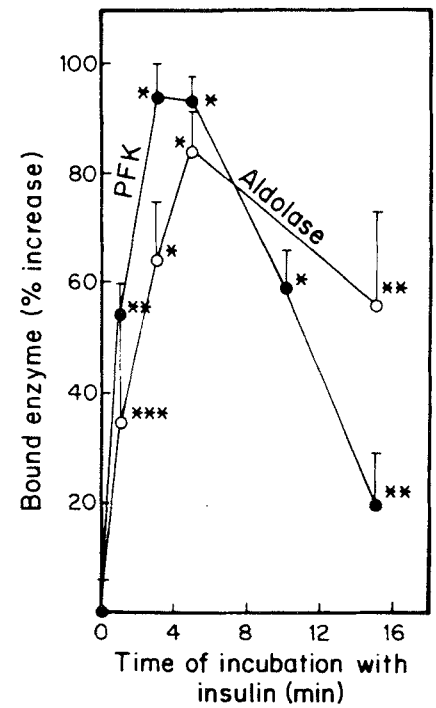

Figure 3 Time-response curves of the effect of insulin on the binding of PFK and aldolase to cytoskeleton in C-6 glial cells. Cells were incubated in the absence and presence of 27 mu/ml insulin for different times. Each point is the mean ± SEM for 6–12 experiments. $*P<0.005$, $**P<0.01$, $***P<0.05$

In the experiments shown in Figures 4 and 5, we studied the possibility that calmodulin may be involved in the insulin-stimulated binding of glycolytic enzymes to cytoskeleton, by using calmodulin antagonists, trifluoperazine (TFP), thioridazine (TRZ) or CGS 9343B (the potent and selective inhibitor of calmodulin activity (Norman *et al.*, 1987). TFP, TRZ or CGS 9343B prevented the insulin-induced increase in cytoskeleton-bound PFK (Figure 4) and aldolase (Figure 5) in C-6 glial cells. The calmodulin antagonists alone had no effect on the enzymes. The concentration of the calmodulin antagonists used in these experiments (Figures 4 and 5) was selected from dose-response curves (not shown) to obtain a maximal effect.

Discussion

The present results reveal that insulin rapidly and transiently stimulates binding of PFK and aldolase to C-6 glial cell cytoskeleton (Figures 2 and 3). The increase in cytoskeleton-bound glycolytic enzymes induced by insulin was prevented by treatment with calmodulin antagonists (Figures 4 and 5), which strongly suggests that Ca^{2+} and calmodulin are involved in this effect of insulin.

Insulin receptor kinase was reported to phosphorylate PFK, and this phosphorylation paralleled autophosphorylation of the β-subunit of the insulin receptor (Sale *et al.*, 1987). Phosphorylation of PFK was shown to increase its affinity to bind to actin (Luther & Lee, 1986), this phosphorylation of PFK may mediate the insulin-stimulated binding of the enzyme to glial cytoskeleton shown here (Figures 2 and 3). The time response curves reveal that the stimulatory effect of insulin on cytoskeleton glycolytic enzymes was very rapid (occurring within minutes) and transient (Figure 3). Insulin was also reported to induce very rapid effects on the cytoskeleton (see reviews, Almas *et al.*, 1992; Kirkeeide *et al.*, 1993), which may also contribute to the increase in glycolytic enzyme binding shown here.

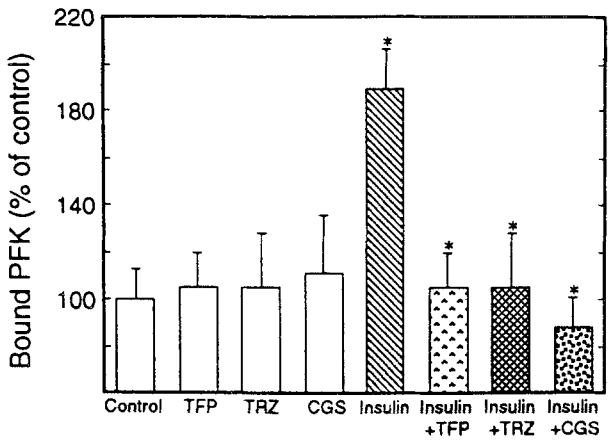

Figure 4 The antagonistic effect of TFP, TRZ and CGS 9343B to the action of insulin on the binding of PFK to cytoskeleton, in C-6 glial cells. Cells were incubated for 30 min in the absence or presence of TFP (25 μM), TRZ (50 μM) or CGS 9343B (10 μM); then insulin (27 mu/ml) was added and incubation was continued for 5 min. 100% activity of bound PFK refers to 2.14 ± 0.30 (mu/mg protein). Values are means ± SEM for 5–6 experiments. $*P<0.005$ (insulin vs control, or insulin + calmodulin antagonists vs insulin)

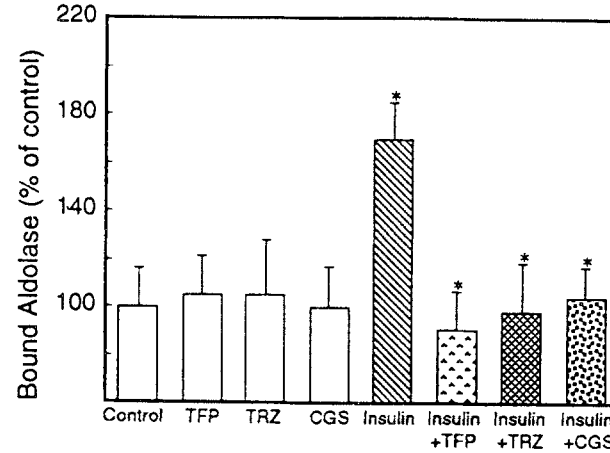

Figure 5 The antagonistic effect of TFP, TRZ and CGS 9343B to the action of insulin on the binding of aldolase to cytoskeleton, in C-6 glial cells. Cells were incubated for 30 min in the absence or presence of TFP (25 μM), TRZ (50 μM) or CGS 9343B (10 μM), then insulin (27 mu/ml) was added and incubation was continued for 5 min. 100% activity of bound aldolase refers to 7.82 ± 1.17 (mu/mg protein). Values are the mean ± SEM for eight experiments. $*P<0.005$ (insulin vs control, or insulin + calmodulin antagonists vs insulin)

There is substantial evidence that calcium and calmodulin are involved in the cellular mechanism of insulin action (Shechter, 1984; Delfert *et al.*, 1988; Draznin, 1988; Chen-Zion *et al.*, 1992b). Ca^{2+} and calmodulin control the dynamic rearrangement of the cytoskeleton (Bennett & Weeds, 1986) as well as the binding of glycolytic enzymes to cytoskeleton (Lilling & Beitner, 1990; Chen-Zion *et al.*, 1992b, 1993; Beitner & Lilling, 1993; Livnat *et al.*, 1993a, 1994; see review, Beitner, 1993). Here we show that calmodulin antagonists trifluoperazine, thioridazine and CGS 9343B, which is a potent and selective inhibitor of calmodulin activity (Norman *et al.*, 1987), prevented the increase in binding of PFK and aldolase to glial cytoskeleton induced by insulin. The mechanism(s) through which Ca^{2+} and calmodulin exert these effects remains to be elucidated. The rapid and transient stimulation of glycolytic enzymes' binding to cytoskeleton, correlates with the rapid and transient simulatory effect of insulin on various protein phosphorylation reactions reported by many investigators. Phosphorylation of various cytoskeletal proteins is known to regulate the dynamic changes in the cytoskeleton in which Ca^{2+}-calmodulin plays a critical role (Nairn & Aderem, 1992; Rasmussen & Means, 1992; Tifaró *et al.*, 1992; Gnegy, 1993). Calmodulin was found to be translocated in cell (Gnegy, 1993), which could alter calmodulin response and Ca^{2+} sensitivity in a select subcellular location.

Insulin (Mohan *et al.*, 1989), as well as Ca^{2+}, Ca^{2+} mobilizing hormones (Bassukevitz *et al.*, 1992), and growth factors, through calmodulin (Bassukevitz *et al.*, 1992; Chen-Zion *et al.*, 1992b) also rapidly and transiently stimulate the binding of hexokinase to mitochondria, where it is linked to oxidative phosphorylation (Gots *et al.*, 1972; Viitanen *et al.*, 1984; Adams *et al.*, 1991). Our previous work in muscle indicated that mitochondrial ATP is required for both the preservation of the basal levels of cytoskeleton-bound glycolytic enzymes and cell structure, as well as for the expression of the stimulatory action of insulin on glycolytic enzymes binding to cytoskeleton (Livnat *et al.*, 1993b).

In summary, the present results are the first demonstration showing that in glial cells, insulin rapidly stimulates binding of glycolytic enzymes to cytoskeleton, and suggest that Ca^{2+} and calmodulin are involved in this stimulatory effect. We have previously found that in muscle, insulin, growth factors and physiological increase in intracellular Ca^{2+}, through calmodulin, also rapidly and transiently stimulate binding of glycolytic enzymes to muscle cytoskeleton (Chen-Zion *et al.*, 1992a, b, 1993; Livnat *et al.*, 1993a, 1994). The present and previous results suggest that acceleration in binding of glycolytic enzymes to cytoskeleton may be a general mechanism in signal transduction of insulin, growth factors and, most probably, other Ca^{2+}-mobilizing hormones, in different tissues. The possible physiological significance of this rapid action is to provide local ATP, produced through the accelerated cytoskeletal glycolysis, to be used in the rapid membrane-cytoskeleton rearrangements following the binding of hormone to its receptor.

Materials and methods

Materials

Insulin from bovine pancreas (approximately 27 I.U. per mg) crystalline was purchased from Sigma Chemical Co. Trifluoperazine dihydrochloride (TFP) was obtained from ICN Flow, UK. Thioridazine hydrochloride was from Taro Pharmaceutical Co., Inc., Haifa, Israel. CGS 9343B was obtained from Ciba-Geigy Corporation, Summit, New Jersey. Other chemicals and enzymes were purchased either from Sigma Chemical Co. or from Boehringer Mannheim GmbH.

Cell cultures

All experiments were performed on C-6 rat glial cells (2B clone). Cells were initially plated in medium containing Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) on uncoated 100 mm Petri dishes (LUX) at density of 0.5×10^6 cells/dish. Cultures were incubated at 37°C in humidified atmosphere of 7.5% CO_2 in air.

Treatment of cultures

Before experiments confluent cultures were transferred to RPMI 1640 medium. It was important to use a serum-free medium that would not contain any hormonal or other factors provided by other defined medium or by serum. After 24 h, cells were washed twice with PBS. Then, cells were incubated at 37°C in PBS containing 5 mM glucose in the absence and presence of insulin and/or calmodulin inhibitors

for different times (as indicated in the figures). All reactions were stopped by adding three vol of ice cold PBS and placing the samples on ice. The cells were then removed with a rubber policeman and pelleted by centrifugation at 150 *g* for 7 min.

Separation and assay of bound enzymes

The particulate (cytoskeleton-bound) PFK or aldolase were separated as follows: the cells were homogenized in a Potter homogenizer for 90 s in 0.25 ml of ice cold 0.25 M sucrose containing 1 mM dithiotreitol and 20 mM NaF, pH 7.5. The homogenate was centrifuged at 4°C for 5 min at 100 *g* and the pellet was discarded. The supernatant was centrifuged at 4°C for 15 min at 27 000 *g*. The 27 000 *g* pellet which was redissolved in 0.2 ml homogenizing solution, is referred to as 'bound fraction'. Cytoskeleton-bound PFK or aldolase was assayed as described previously (Lilling & Beitner, 1990). PFK was assayed under maximal (optimal) conditions (pH 8.2), by coupling with aldolase, triose-phosphate isomerase and α-glycerophosphate dehydrogenase. After the addition of fructose 6-phosphate, the rate of disappearance of NADH was measured spectrophotometrically. Aldolase activity was assayed after addition of fructose 1,6-P_2 by coupling with triose-phosphate isomerase and α-glycerophosphate dehydrogenase.

Electron microscopy of the 'bound fraction'

The 'bound fraction' was diluted 10-fold for microscopy in homogenizing solution. Samples were stained with 1% uranyl acetate for 2 min prior to viewing. The samples were examined by a Jeol 1200 Ex electron microscope.

Protein determination

Samples for protein determination were taken after the first homogenization of the cells. Protein was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard.

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