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Sub-compartmentation of the 'cytosolic' glucose 6-phosphate pool in cultured rat hepatocytes

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[^{14}C]Glucose release either from endogenous ^{14}C-prelabelled glycogen or from added ^{14}C-labelled glucose 6-phosphate was measured in filipin-treated, permeabilized hepatocytes in 48 h culture. [^{14}C]Glucose output from prelabelled glycogen was not altered by the addition of 5 mM glucose 6-phospate to the incubation medium. Conversely, [^{14}C]glucose release from 5 mM labelled glucose 6-phosphate was not influenced by different glycogen concentrations in the cells. Moreover, in the permeabilized cells the anion transport inhibitor DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) inhibited only the liberation of [^{14}C]glucose from labelled glucose 6-phosphate but not from glycogen. It is therefore concluded that there exist at least 2 separate, mutually non-accessible glucose 6-phosphate pools in cultured rat hepatocytes, one linked to glycogenolysis and the other to gluconeogenesis.

Glucose 6-phosphate; Glucokinase; Glycogenolysis; Gluconeogenesis; Glycolysis; Filipin

1. INTRODUCTION

Net glucose release or uptake by liver cells is generally believed to depend on the rate of cycling between glucose and glucose 6-phosphate, i.e. on the actual rates of the glucokinase and glucose-6-phosphatase reactions. It appears to be widely accepted that the rates of the two enzymes are regulated only by the cytosolic concentrations of the substrates glucose and glucose 6-phosphate [1]. However, a number of studies have indicated that the enzymes might be under direct allosteric or hormonal regulation [2–6]. Moreover, it is the predominant view that glucose 6-phosphate as all other intermediates of metabolism form homogeneous pools in their respective subcellular compartments such as the cytosol, the mitochondrial matrix or the intralysosomal space. This belief has been and still is the basis of innumerable studies measuring metabolite and effector levels in order to gain insight into the mechanisms of metabolic regulation.

In the classical view glucose 6-phosphate constitutes a major crossroad of cytosolic metabolism: glucose uptake and release, glycogen synthesis and degradation; gluconeogenesis and glycolysis as well as the pentose phosphate pathway intersect at glucose 6-phosphate. Thus, homogeneity or inhomogeneity of this pool is of the utmost importance for the understanding of carbohydrate metabolism. Here, this problem was studied using permeabilized hepatocytes in culture by measuring the influence of exogenous glucose 6-phosphate on the liberation of glucose from endogenous glycogen and conversely of endogenous glycogen on the formation of glucose from exogenous glucose 6-phosphate. From the results, it is concluded that in hepatocytes glycogenolysis and e.g. gluconeogenesis operate through separate glucose 6-phosphate pools.

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2. MATERIALS AND METHODS

2.1. *Materials*

Chemicals were of analytical grade and pur-

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chased from commercial sources. Filipin and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were from Sigma (Taufkirchen, FRG). Radiochemicals were supplied from Amersham Buchler (Braunschweig, FRG). α-Hemolysin (= α-toxin) from *Staphylococcus aureus* was a kind gift from Behring Werke (Marburg, FRG).

2.2. *Cell culture and permeabilization*

Hepatocytes were isolated from fed male Wistar rats (200–250 g) by collagenase perfusion and cultured in medium M199 essentially as in [6] with medium changes at 4 and 24 h. In order to induce different glycogen contents, the cells were cultured in the presence of either 5 or 25 mM glucose from 24 to 48 h. This pretreatment yielded glycogen contents of 16 μmol glycogen-glucose/g wet wt (low glycogen) or 150 μmol glycogen-glucose/g wet wt (high glycogen), respectively. Glycogen was prelabelled by supplying the cell cultures with [U-^{14}C]glucose (60 kBq/ml = 1.6 μCi/ml corresponding to 700000 or 140000 dpm/μmol, respectively) from 24 to 48 h of culture. After 48 h the culture medium was sucked off and cells were washed once with Hepes buffer (20 mM, pH 7.4) containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$ and 1% BSA (buffer A). Subsequently the cells (25 mg/dish) were permeabilized routinely by incubation in 2.5 ml buffer A with 50 μM filipin for 1 min at 37°C (cf. [7]). Afterwards the cells were washed twice with prewarmed buffer A and then incubated in buffer A containing substrates or inhibitors as indicated. Permeabilization with 45 U/ml α-toxin was performed at 37°C for 5 min in some control experiments essentially as described [8].

2.3. *Assays*

In two series of experiments glucose release from the cells was determined either from prelabelled [U-^{14}C]glycogen or from 5 mM [U-^{14}C]glucose 6-phosphate (7.4 kBq/ml = 0.2 μCi/ml corresponding to 88000 dpm/μmol) in cultures run in parallel. Incubations were carried out in 2.5 ml of medium for up to 20 min as indicated. 100-μl samples were taken from the incubation medium and applied to a column of Dowex formate 1 × 8, 200 × 400 mesh (0.5 × 2 cm) to separate radioactive glucose and glucose 6-phosphate. Glucose was eluted from the column by 2 ml water and glucose 6-phosphate subsequently in another 2 ml of 0.4 M sodium formate. Radioactivity in the effluents was counted in 15 ml Quickscint 2000 from Zinsser (Frankfurt) in a Philips PW 4700 scintillation counter. Product formation was calculated by dividing the total radioactivity of the product by the specific activity of the labelled substrate [6].

3. RESULTS

3.1. *Permeabilization of cultured hepatocytes*

Filipin, digitonin and saponin, which complex with membrane-bound cholesterol [7,9,10], and α-hemolysin (= α-toxin from *S. aureus*), which forms pores in biomembranes and liposomes [8], have been used to permeabilize cells. These agents can be expected to permeabilize plasma membranes preferentially, because intracellular membranes such as the endoplasmic reticulum and the inner mitochondrial membrane have a lower cholesterol content, or since α-hemolysin, respectively, should reach intracellular membranes only in cells with partly disrupted plasma membranes, which cannot be avoided in the experimental preparations. Thus, cultured rat hepatocytes were permeabilized at 37°C by treatment with 50 μM filipin for 1 min or, for comparison, with 45 U/ml α-toxin for 5 min. These procedures led to permeabilization of 95–100% of the cells as estimated by trypan blue uptake. Leakage of lactate dehydrogenase (LDH) was measured during a subsequent incubation of the cells in the respective permeabilization buffer without filipin or α-toxin for 20 min and found not to exceed 20% of total LDH with both methods. The kinetics of microsomal glucose-6-phosphatase in the permeabilized cells were determined and compared to those in a normal microsomal preparation. They were found to be similar with a half-saturation concentration for glucose 6-phosphate at pH 7.4 in the range of 5 mM (not shown; cf. however fig.1), which indicated that both filipin and α-toxin left the endoplasmic reticulum (ER) essentially intact. This conclusion was corroborated by the finding that the glucose-6-phosphatase system, which is composed of a glucose 6-phosphate translocator in the ER membrane, the glucose-6-phosphate hydrolase proper facing the ER lumen and a phosphate and glucose translocator in the ER

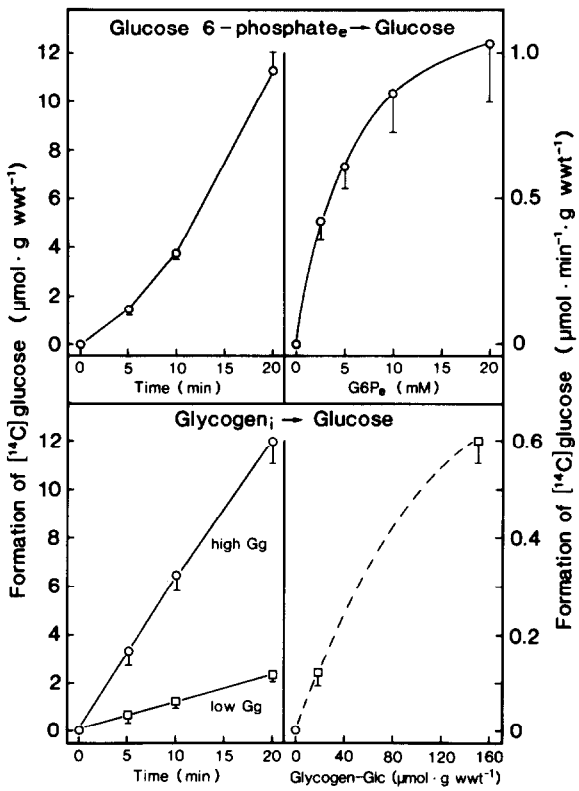

Fig.1. Dependence on time and substrate concentration of [^{14}C]glucose formation from exogenous glucose 6-phosphate (upper) and from endogenous labelled glycogen (lower) in permeabilized cultured rat hepatocytes. Cells were cultured for 48 h in the presence of 5 mM glucose (upper) and 5 or 25 mM [U-^{14}C]glucose (lower) and then permeabilized with filipin. Filipin was removed by washing twice in buffer A. In the upper panel the reaction was then started with new buffer A containing 5 mM [U-^{14}C]glucose 6-phosphate (left) or the indicated [U-^{14}C]glucose 6-phosphate concentration (right) and continued for the indicated time periods (left) or for 20 min (right). In the lower panel the reaction was started with new buffer A and continued for the indicated time periods (left) or for 20 min (right). Values represent means ± SE from three separate cultures, each run in duplicate. Gg, glycogen; G6P, glucose 6-phosphate; Glc, glucose; e, exogenous; i, intracellular/endogenous.

membrane [11,12], could be inhibited by an anion transport inhibitor (cf. fig.4). Thus, it appeared permissible to conduct all further studies with filipin-permeabilized cells.

3.2. *Glucose formation from exogenous glucose 6-phosphate and endogenous glycogen*

In filipin-permeabilized hepatocytes in culture [^{14}C]glucose release from [^{14}C]glucose 6-phosphate was linear with time and showed normal saturation kinetics (fig.1, upper panels); [^{14}C]glucose formation from [^{14}C]glycogen was also linear with time and dependent on the intracellular glycogen level (fig.1, lower panel). The regulation of glucose output by the liver depends ultimately on the relative rates of the glucose-6-phosphatase and the glucokinase reactions. According to present understanding the rate of the glucose-6-phosphatase reaction is dependent on the concentration of a homogeneous cytosolic glucose 6-phosphate pool, which is replenished by glycogenolysis,

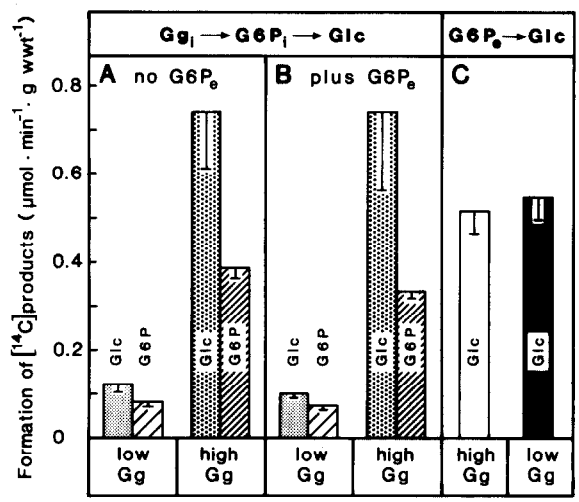

Fig.2. Formation of [^{14}C]glucose and [^{14}C]glucose 6-phosphate from labelled endogenous glycogen (A,B) or exogenous labelled glucose 6-phosphate (C) in permeabilized cultured rat hepatocytes. Cells were cultured for 48 h in the presence of 5 or 25 mM glucose in order to induce low or high glycogen levels, respectively. Cells were then permeabilized with filipin. After two washings with buffer A the reaction was started as described in fig.1 and run for 20 min. If labelled glycogen was the substrate for the formation of glucose and glucose 6-phosphate, the reaction was run in either the absence (A) or presence (B) of 5 mM unlabelled glucose 6-phosphate. If exogenous labelled glucose 6-phosphate was the substrate (C) for glucose formation, it was present at a concentration of 5 mM. Values are means ± SE of four different cultures run in duplicate. Abbreviations as in fig.1.

glycogenolysis, gluconeogenesis and glucose phosphorylation and which is depleted by glycogen synthesis, glycolysis, glucose liberation and pentose phosphate formation. If this assumption is true, it should be possible in permeabilized hepatocytes to dilute [^{14}C]glucose formation from labelled endogenous glycogen by exogenously added glucose 6-phosphate and, conversely, to dilute [^{14}C]glucose liberation from exogenous labelled glucose 6-phosphate by glucose 6-phosphate formed from different levels of endogenous glycogen. This, however, was not the case.

[^{14}C]Glucose formation from labelled endogenous glycogen, at both low and high levels, was the same in the absence or presence of 5 mM exogenous glucose 6-phosphate (fig.2A vs B). Also, the amount of [^{14}C]glucose 6-phosphate formed from labelled glycogen was not altered by the addition of exogenous glucose 6-phosphate (fig.2A vs B). Conversely, the formation of [^{14}C]glucose from labelled exogenous glucose 6-phosphate was the same, when the rate of glucose 6-phosphate formation from endogenous glycogen was low or high depending on the glycogen concentration (fig.2C). These data indicate that there must be at least two glucose 6-phosphate pools, one of which is replenished by glycogenolysis and which is not accessible to exogenously added glucose 6-phosphate and the other, which is replenished by probably gluconeogenesis and is not accessible to glucose 6-phosphate formed endogenously from glycogen.

3.3. *Inhibition of glucose formation from exogenous glucose 6-phosphate, but not from endogenous glycogen by DIDS*

At physiological pH glucose 6-phosphate is a dianion. Its transport from the cytosol into the lumen of the endoplasmic reticulum can be inhibited by anion transport inhibitors such as DIDS, which competes for the active site of the glucose 6-phosphate translocator [13]. In the filipin-permeabilized hepatocytes 1 mM DIDS inhibited the formation of [^{14}C]glucose from exogenous labelled glucose 6-phosphate but not from endogenous labelled glycogen (fig.3). It must be concluded from these findings either that the glucose 6-phosphate translocator involved in the liberation of glucose from glycogen is different from the translocator involved in the conversion of

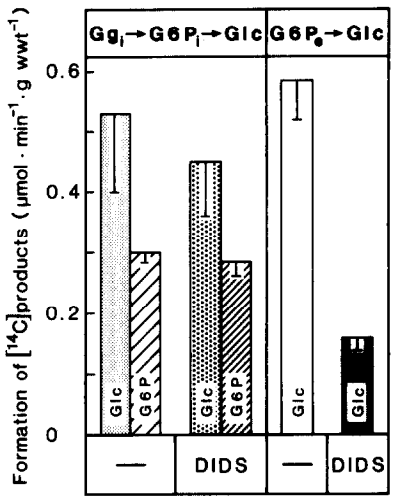

Fig.3. Effect of the anion transport inhibitor DIDS on [^{14}C]glucose formation from endogenous labelled glycogen or exogenous labelled glucose 6-phosphate in permeabilized rat hepatocytes. Cells were cultured for 48 h in the presence of 25 mM glucose, which was labelled, if glycogen was to be the substrate. After two washings in buffer A the reaction was started by adding new buffer A containing 5 mM glucose 6-phosphate, which was labelled, if it was to be the substrate, and 1 mM DIDS, where indicated. Values are means ± SE of three separate cultures run in duplicate. Abbreviations, see fig.1.

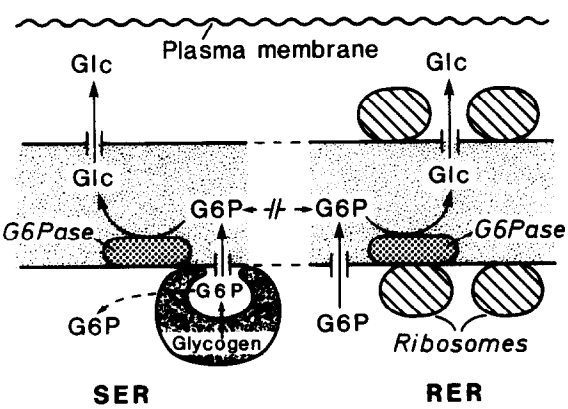

Fig.4. Scheme of the glucose-6-phosphatase system linked to glycogenolysis in the SER and probably to gluconeogenesis in the RER. Glc, glucose; G6P, glucose 6-phosphate; S(R)ER, smooth (rough) endoplasmic reticulum.

exogenous glucose 6-phosphate to glucose, which is very unlikely, or that the translocator involved in the conversion of glycogen to glucose was not accessible to the inhibitor, which would be very plausible, if it were shielded by glycogen particles adhering to the endoplasmic reticulum (fig.4).

4. DISCUSSION

It was shown in the present investigation that in permeabilized cultured rat hepatocytes [^{14}C]-glucose release from labelled glycogen could not be inhibited by dilution with exogenous glucose 6-phosphate and, conversely, that [^{14}C]glucose formation from exogenous labelled glucose 6-phosphate could not be altered by dilution with glucose 6-phosphate originating from glycogen breakdown (fig.2), and furthermore, that only the formation of [^{14}C]glucose from exogenous labelled glucose 6-phosphate but not from endogenous labelled glycogen could be inhibited by the anion transport inhibitor DIDS (fig.3).

4.1. *Sub-compartmentation of the cytosolic glucose 6-phosphate pool*

The findings of the present study are incompatible with a homogeneous glucose 6-phosphate pool linking such diverse processes as glucose uptake and release, glycogen synthesis and degradation, glycolysis and gluconeogenesis as well as pentose phosphate formation. It must be concluded that there exist at least two different, mutually non-accessible glucose 6-phosphate pools. One of these is apparently involved in glycogenolysis: it is quite feasible that glycogen particles containing all enzymes required for glycogen degradation [14] are linked to the smooth endoplasmic reticulum [15,16] in such a way that the glucose 6-phosphate formed cannot exit freely from the 'glycogen subcompartment' to the cytosol but only via the glucose 6-phosphate translocator to the lumen of the reticulum (fig.4). Some leakage of glucose 6-phosphate to the cytosol may be possible; entry of glucose 6-phosphate from the cytosol to the glycogen subcompartment is, however, not possible. The second glucose 6-phosphate pool might be linked to gluconeogenesis; it may be freely or partly miscible with the glucose 6-phosphate pool which is filled by glucose phosphorylation and emptied by glycogen synthesis, glycolysis and pentose phosphate formation. This pool may have access to the glucose-6-phosphatase system predominantly in the glycogen-free rough endoplasmic reticulum but also in the glycogen-rich smooth endoplasmic reticulum [14–16] (fig.4). The present findings also allow the conclusion that the glucose 6-phosphate derived from glycogen breakdown and that originating from gluconeogenesis do not mix in the lumen of the endoplasmic reticulum either (fig.4).

In an in vivo study in rats of the conversion of [U-^{14}C]fructose to glucose evidence was obtained which was interpreted to show that the hepatic glucose 6-phosphate pool was compartmentalized between gluconeogenesis and glycolysis [17]. In another recent in vivo investigation with rats the findings obtained were interpreted to show compartmentation of the UDP-glucose pool between glycogen synthesis and glucuronidation reactions [18].

4.2. *Regulatory significance of the different cytosolic glucose 6-phosphate pools*

The relative sizes of the glucose 6-phosphate pool linked to glycogenolysis and of that probably linked to gluconeogenesis are unknown; however, it can be assumed that neither is so much smaller than the other that it can be neglected. Thus, it is presently impossible to calculate the respective pool size from the usual overall measurement of the glucose 6-phosphate level.

The glucose-6-phosphatase system linked to glycogenolysis need not be controlled directly; it would be sufficient if it were controlled indirectly by the activation or inactivation of glycogen breakdown through the hormonal [19] or nervous [20,21] system. In contrast, the glucose-6-phosphatase system linked probably to gluconeogenesis can be expected to be under the control of both substrate availability and of the hormonal and nervous system. It has been shown recently that liver glycogen is formed primarily from three-carbon substrates via gluconeogenesis and also to a lesser extent directly from glucose [22–25], and that glycogen synthesis from pyruvate is located in the periportal and from glucose in the perivenous zone of the liver parenchyma [26]. Thus, in the periportal zone the glucose-6-phosphatase system should be regulated to control substrate flux at the glucose 6-phosphate branch point to glucose or glycogen

formation; in the perivenous zone the glucose-6-phosphatase system should be regulated to avoid excessive recycling of glucose 6-phosphate just formed from glucose in order to direct carbon flow to glycogen synthesis and glycolysis. Evidence for a negative control of the glucose-6-phosphatase system by insulin has indeed been presented [3–6].

It has been shown recently with isolated microsomes from rat liver that glucose 6-phosphate stimulates the ATP-dependent uptake of Ca^{2+} into the endoplasmic reticulum [27,28]. It has been suggested that α-adrenergic stimulation of glycogenolysis via an increase of cytosolic Ca^{2+} needs to be controlled by the ATP-dependent removal of Ca^{2+} into the endoplasmic reticulum, where it is to be co-precipitated with P_i generated by glucose 6-phosphate hydrolysis. The conclusion of the present study that glycogenolysis and glucose 6-phosphate hydrolysis in the area of the SER are directly linked would be in line with the proposed role of glucose-6-phosphatase in the microsomal Ca^{2+}-sequestering system.

4.3. *Conclusion*

This study has shown that the cytosolic glucose 6-phosphate pool is sub-compartmentalized at least into two parts. Calculation of metabolic rates based on the measured overall activity of glucose-6-phosphatase and on the determined overall concentration of glucose 6-phosphate no longer seems permissible.

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