Calcium activates glucose-6-phosphatase in intact rat hepatic microsomes

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The effects of Ca^{2+} on the microsomal glucose-6-phosphatase activity were investigated. Evidence is provided that increases by Ca^{2+} in both the pyrophosphatase and the glucose-6-phosphate-hydrolysing activities are due to an increase in microsomal transport capacity of T_2 , the phosphate/pyrophosphate-transport protein.

INTRODUCTION

Hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9) is a multi-component system consisting of at least five different polypeptides: the glucose-6-phosphatase enzyme with its active site located on the luminal side of the endoplasmic reticulum, a Ca^{2+} -binding protein termed stabilizing protein, and three transport proteins, T_1 , T_2 and T_3 , which facilitate the movement of glucose 6-phosphate, pyrophosphate (and phosphate) and glucose respectively across the endoplasmic-reticulum membrane (Fig. 1). The absence of any of these protein components will impair glucose 6-phosphate hydrolysis and hepatic glucose production, as demonstrated by the hypoglycaemia in the genetic deficiencies of the proteins in types 1a, 1aSP, 1b and 1c glycogen-storage disease (Cori & Cori, 1952; Narisawa *et al.*, 1978; Lange *et al.*, 1980; Burchell *et al.*, 1987, 1989a,b; Burchell & Waddell, 1990).

The role of Ca^{2+} as a second messenger in glycogen metabolism has been widely studied, both in conjunction with (Cohen, 1983) and independently of cyclic AMP (Mine *et al.*, 1989). The stabilizing protein of the glucose-6-phosphatase system is essential for glucose-6-phosphatase activity *in vivo* and *in vitro* (Burchell *et al.*, 1985; Burchell & Waddell, 1990), and it is a 21 kDa binding protein with a micromolar K_D value for Ca^{2+} . However, nothing is known about the effects of Ca^{2+} binding to the stabilizing protein on glucose-6-phosphatase activity. Recently it has been reported that physiological concentrations of Ca^{2+} inhibit the glucose-6-phosphatase transport protein T_1

in permeabilized hepatocytes and in intact microsomes (van de Werve, 1989). The inhibition, which it was suggested may be due to the Ca^{2+} -binding capacity of the 21 kDa stabilizing protein, directly contradicts earlier reports (Swanson, 1950; Nordlie & Sukalski, 1985), which demonstrated that the glucose-6-phosphatase system was essentially unaffected by Ca^{2+} . Slight inhibition of glucose-6-phosphatase activity by high concentrations of Ca^{2+} and Mg^{2+} has been reported (Beaufay & de Duve, 1954; Nordlie & Arion, 1965). Since the rate of glycogenolysis in liver is greatly increased by the release of intracellular Ca^{2+} (Cohen, 1983), it seems illogical that glucose-6-phosphatase, the terminal step of glycogen breakdown, should be thus inhibited. We therefore reinvestigated the effects of Ca^{2+} on the glucose-6-phosphatase system.

MATERIALS AND METHODS

Materials

Glucose 6-phosphate (monosodium salt) was from BDH, Poole, Dorset, U.K.; mannose 6-phosphate (disodium salt) and histone 2A were purchased from Sigma Chemical Co., Poole, Dorset, U.K. HEPES and sodium cacodylate were also from Sigma. Lubrol 12A-9 was obtained from ICI Organics Division, Manchester, U.K. All other chemicals were of analytical-reagent grade.

Preparation of hepatic microsomes

Microsomes were prepared from livers of male Wistar rats in buffered 0.25 M-sucrose/5 mM-HEPES (pH 7.4) as previously described (Countaway *et al.*, 1988). Diabetes was induced by a single tail-vein injection of streptozotocin (75 mg/kg body wt.) in buffered citrate, pH 4.5. Only animals in which the development of diabetes was confirmed 48 h later, by measurement of blood sugar concentration, were used (Burchell & Cain, 1985). Starved-rat liver microsomes were prepared after a 16 h fast.

Assays

Glucose-6-phosphatase, pyrophosphatase and mannose-6-phosphatase activities were assayed and calculated as in Burchell *et al.* (1988), except that EDTA was omitted from all assays. All activities are expressed as μmol of P_i released/min per mg of microsomal protein. Microsomes isolated from liver homogenates and not treated further before assay are referred to as untreated, and are a mixture of intact and disrupted microsomes. The proportion of intact microsomes was determined by assays

Fig. 1. The hepatic microsomal glucose-6-phosphatase system

Key: G, glucose; G-6-P, glucose 6-phosphate; SP, stabilizing protein; ER, endoplasmic reticulum.

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of low- K_m mannose-6-phosphatase activity, which is only expressed in disrupted microsomes (Arion *et al.*, 1976). All the microsomal preparations used in the present paper were > 95 % intact. All the 'undisrupted' values reported in the present paper are 'intact' values, which eliminates the large errors in activity measurements that can occur if even a small proportion of microsomes are disrupted. To determine the effects of bivalent cations, assays were carried out as above in the presence of the chloride salt of the appropriate cation.

P values were calculated from t tests with pooled analysis of variance by using Microstat (Release 4.0) software (Ecosoft, IN, U.S.A.).

The direct transport assay for glucose 6-phosphate and pyrophosphate uptake in starved-rat liver microsomes was carried out with 1 mM of the appropriate substrate exactly as described in Waddell *et al.* (1989). Protein concentrations were determined by the method of Peterson (1977).

RESULTS AND DISCUSSION

van de Werve (1989) compared glucose-6-phosphatase activity in a 25 mM-EGTA buffer with activity in a 25 mM-EGTA buffer plus various amounts of free Ca^{2+} . In an attempt to carry out experiments analogous to those reported by van de Werve (1989), we used an EGTA buffer system to maintain constant levels of micromolar free Ca^{2+} . Unfortunately, in control experiments we found that the EGTA itself had two effects on the glucose-6-phosphatase system. Firstly, it caused disruption of the microsomal membrane; secondly, it caused loss of enzyme activity in disrupted microsomes. The combination of the two effects has complex effects on glucose-6-phosphatase activity. The transport proteins are rate-limiting to glucose-6-phosphatase activity in intact microsomes, so the increased disruption leads to an apparent increase in activity in untreated microsomes. In contrast, in disrupted microsomes a decrease in activity is seen. For example, when 1 mM-mannose 6-phosphate is used to measure intactness in starved-rat liver microsomes, the intactness of the microsomal membrane was 96 %, 75 %, 72 % and 65 % in the presence of 0, 2.5, 10 and 25 mM-EGTA respectively, whereas the activity of the glucose-6-phosphatase enzyme in disrupted microsomes under the same conditions fell from 100 % to 80 %, 59 % and 40 % respectively.

The complex effects of EGTA on glucose-6-phosphatase activity mean that EGTA cannot be used as a buffer to study the effects of Ca^{2+} , especially on the transport protein, as the microsomal disruption makes accurate measurement of transport capacity extremely difficult. Nitrilotriacetic acid systems are also often used to maintain free Ca^{2+} levels (Scott *et al.*, 1980), but again we found that nitrilotriacetic acid inhibited glucose-6-phosphatase activity in disrupted microsomes and caused disruption of intact microsomes in a very similar manner to EGTA. A wide range of other metal-binding agents are well known to inhibit glucose-6-phosphatase activity (for an early review see Nordlie, 1971). We therefore looked at the effects of adding a wide range of Ca^{2+} concentrations to microsomal samples in the absence of metal-binding agents.

The glucose-6-phosphatase activity in intact microsomes is a measure of the combined rates of the glucose-6-phosphatase enzyme and the three transport proteins (Arion *et al.*, 1980). The pyrophosphatase activity in intact microsomes, however, is an estimate of the combined rates of the glucose-6-phosphatase enzyme activity and only one transporter, T_2 (Arion *et al.*, 1980). Table 1 shows the results of glucose-6-phosphatase and pyrophosphatase assays carried out on intact microsomes from fed, starved and diabetic rats in the absence or presence of 1 mM- Ca^{2+} . The results demonstrate that Ca^{2+} activates both the

Table 1. Glucose-6-phosphatase activity in intact liver microsomes in the presence of Ca^{2+}

Values represent means $\pm$ S.E.M. of eight experiments. The average intactness of the preparations from fed, starved and diabetic rats was 95 %, 97 % and 93 % respectively. Each experiment comprised at least six rat livers. Differences calculated from values with no added Ca^{2+} : * $P < 0.01$.

(a) Pyrophosphate as substrate			
Rat liver microsomes	Ca^{2+} (mM)	V_{max} ($\mu\text{mol}/\text{min}$ per mg)	K_m (mM)
Fed	—	0.06 ± 0.01	2.5 ± 0.3
	+1	0.08 ± 0.01	$1.4 \pm 0.1^*$
Starved	—	0.09 ± 0.02	3.5 ± 0.2
	+1	0.09 ± 0.01	$1.1 \pm 0.2^*$
Diabetic	—	0.09 ± 0.03	4.5 ± 0.2
	+1	0.12 ± 0.01	$1.2 \pm 0.3^*$

(b) Glucose 6-phosphate as substrate			
	Ca^{2+} (mM)	V_{max} ($\mu\text{mol}/\text{min}$ per mg)	K_m (mM)
Fed	—	0.14 ± 0.02	3.2 ± 0.3
	+1	0.20 ± 0.01	$1.2 \pm 0.1^*$
Starved	—	0.25 ± 0.03	3.4 ± 0.3
	+1	0.36 ± 0.02	$1.0 \pm 0.2^*$
Diabetic	—	0.30 ± 0.02	6.0 ± 0.5
	+1	0.37 ± 0.03	$2.9 \pm 0.2^*$

glucose-6-phosphatase and pyrophosphatase activities of intact microsomes by lowering the K_m , with only a very small effect on V_{max} . Similar experiments demonstrated that identical concentrations of Ca^{2+} had no effect on these activities in detergent-disrupted microsomes. These data indicated that Ca^{2+} ions have a direct activating effect on one or more of the translocases of the glucose-6-phosphatase system, but no effect on the activity of the catalytic subunit of glucose-6-phosphatase.

The greatest activation in intact microsomes is manifest as a decrease in the K_m for pyrophosphatase activity (see Table 1a), showing that Ca^{2+} activates T_2 . Similar but somewhat smaller effects were also observed with glucose 6-phosphate as substrate (Table 1b). T_1 rather than T_2 is thought to be the rate-limiting step in glucose 6-phosphate hydrolysis in intact rat liver microsomes (Arion *et al.*, 1980), which suggests that T_1 as well as T_2 might be activated by Ca^{2+} . We therefore used a micro-filtration assay to measure directly glucose 6-phosphate and pyrophosphate uptake into intact rat liver microsomes in the absence and presence of 1 mM- Ca^{2+} to determine whether both T_1 and T_2 are affected by Ca^{2+} . The 1 mM- Ca^{2+} increased the initial uptake of pyrophosphate by 160 %, whereas its effect on the initial rate of glucose 6-phosphate transport was negligible. The simplest explanation of these results is that only T_2 is activated by Ca^{2+} . Similar activation of transport has been observed in rat liver mitochondria, where incubation in the presence of Ca^{2+} increased the matrix pyrophosphate content by up to 300 % (Davidson & Halestrap, 1987).

When glucose 6-phosphate is the substrate, the role of T_2 is to remove phosphate from the lumen of the endoplasmic reticulum. In type 1c glycogen-storage disease, when T_2 is deficient, phosphate builds up to high levels in the lumen of intact microsomes, which inhibits the glucose-6-phosphatase enzyme, resulting in a higher than normal K_m and a lower than normal V_{max} (Burchell *et al.*, 1987, 1989a,b). If the phosphate concentration in the lumen of the microsomes is normally somewhat inhibitory to the

glucose-6-phosphatase enzyme, an increase in the transport capacity of T_2 would lead to a decrease in K_m and an increase in $V_{\max}$ of glucose-6-phosphatase activity in intact microsomes, which is exactly what occurs in the presence of 1 mM- Ca^{2+} (Table 1b).

Rat liver microsomes bind Ca^{2+} non-specifically (Sampson & Karler, 1963; Sanui & Pale, 1967), which makes it impossible to determine the true Ca^{2+} concentrations which activate T_2 , and we are unable to use any of the buffer systems usually used to control free Ca^{2+} levels. In Table 1 we used 1 mM- Ca^{2+} , which was the concentration that gave the maximum activation, but clearly the concentrations of free Ca^{2+} ions must be very much lower. Experiments where less than 0.5 mM- Ca^{2+} was added externally had no effect on glucose-6-phosphatase activity. At concentrations 10-fold higher than those reported in Table 1 (10 mM), we found significant inhibition of the enzyme system. Although this is in agreement with earlier reports (Beaufay & De Duve, 1954; Nordlie & Arion, 1965), we interpret this inhibition as due to Ca^{2+} aggregation of microsomes (Kamath *et al.*, 1971), which would restrict substrate access, therefore apparently decreasing activity.

Very little is known about the regulation of the phosphate/pyrophosphate translocase T_2 , and this paper provides the first evidence of direct activation of this transport protein.

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