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Liver glucose-6-phosphatase activity is not modulated by physiological intracellular Ca^{2+} concentrations

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1. In the presence of MgATP and increasing amounts of added Ca^{2+}, isolated liver microsomal vesicles accumulate approx. 10 nmol of Ca^{2+}/mg of protein and buffer ambient free Ca^{2+} at increasing concentrations (0.22–10.9 μM). Under these experimental conditions, microsomal glucose-6-phosphatase activity is unaffected by the concentration of extravesicular free Ca^{2+}. 2. Different levels of intravesicular Ca^{2+} were obtained by treating microsomes with the Ca^{2+} ionophore A23187 and by stimulating active microsomal Ca^{2+} accumulation with P_i (3 mM). In both instances, microsomal glucose-6-phosphatase activity is unaffected by the level of intravesicular Ca^{2+}.

INTRODUCTION

Liver microsomal glucose-6-phosphatase (G-6-Pase; EC 3.1.3.9) catalyses the terminal reaction of gluconeogenesis and glycogenolysis [1]. Since the rate of hepatic glycogenolysis is increased by the rise in cytosolic free Ca^{2+} levels [2], it seems logical that G-6-Pase activity might be also modulated by cytosolic free Ca^{2+}. In this respect, previous reports are conflicting. There have been reports from three independent laboratories that Ca^{2+} activates G-6-Pase activity in intact rat liver microsomes [3–5]. In contrast, other authors [6] concluded that free Ca^{2+} inhibits G-6-Pase activity of rat liver microsomes and of permeabilized rat hepatocytes. Discrepancies can be due to the experimental systems employed. In fact, G-6-Pase activity has been measured in the presence of non-physiologically high Ca^{2+} concentrations [4] or alternatively of high concentrations of EGTA in order to lower free Ca^{2+} to cytosol-like sub-micromolar levels [5,6]. In the latter condition, the EGTA–Ca^{2+} complex by itself inhibits microsomal G-6-Pase activity [7].

Against this background we have re-investigated the effect of Ca^{2+} on G-6-Pase activity of intact rat liver microsomes by using a different experimental approach. In particular, experiments have been performed under cytosol-like conditions with respect to the levels of free Ca^{2+} and in the absence of EGTA. To this end, liver microsomes have been allowed to buffer external free Ca^{2+} in the presence of ATP, and their G-6-Pase activity has been evaluated thereafter. Data reported indicate a lack of modulation by sub-micromolar concentration of Ca^{2+} on the rate of hydrolysis of glucose 6-phosphate (G-6-P) by liver microsomal G-6-Pase.

EXPERIMENTAL

Materials

ATP, G-6-P (disodium salt), phosphocreatine and creatine kinase (Sigma type III) were from Sigma, St. Louis, MO, U.S.A. [1-^{14}C]Glucose 6-phosphate (49 mCi/mmol) was from DuPont–New England Nuclear, Dreieich, Germany. Ca^{2+} electrodes were purchased from Ionetics Inc., Palo Alto, CA, U.S.A. All other chemicals were of analytical grade.

Preparation of liver microsomes

Male Sprague–Dawley rats (180–230 g) were used. Liver microsomes were prepared as reported previously [8]. Microsomal fractions were resuspended (approx. 80 mg of protein/ml) in a medium which had the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 5; Mops, 20, pH 7.2. Intactness of the microsomal membrane was ascertained by measuring the latency of mannose-6-phosphatase activity [4]. Mannose-6-phosphatase activity of microsomes was less than 10% of the activity of fully disrupted microsomal vesicles, in all the preparations employed.

Incubation procedures

Microsomes were incubated in a thermostatically regulated (37 °C) Plexiglas vessel in which a Ca^{2+} electrode and a reference electrode (Radiometer K4040) were immersed. The incubation medium (1 ml) was as follows (mM): KCl, 100; NaCl, 20; $MgCl_2$, 5; Mops, 20 (pH 7.2); ATP, 3; phosphocreatine, 10; NaN_3 (as mitochondrial inhibitor), 5. Creatine kinase (10 units/ml) was also present. The Ca^{2+} electrode was calibrated as described elsewhere [9]. The amount of total Ca^{2+} present in the incubation medium before any Ca^{2+} addition (i.e. Ca^{2+} already present as a routine contaminant of solutions) ranged from 9 to 20 nmol/ml, as measured by atomic-absorption spectroscopy.

G-6-Pase assay

G-6-Pase activity was measured by using [^{14}C]G-6-P [10]. Briefly, G-6-P (including trace amounts of [^{14}C]G-6-P) in a small volume of the basic buffer (50 μl) was added to the microsomal suspensions incubated as above. At the selected time points, aliquots (50 μl) of the incubation mixture were transferred into tubes containing 0.5 ml of 0.3 M-$ZnSO_4$. After mixing, 0.5 ml of a saturated solution of $Ba(OH)_2$ was added and tubes were centrifuged to remove white precipitate. A 0.5 ml portion of the clear supernatant was used to measure [^{14}C]glucose produced from [^{14}C]G-6-P, by liquid-scintillation spectroscopy. More than 95% of glucose was recovered in the clear supernatant, as assessed by using standard [^{14}C]glucose. Also, contamination of the clear supernatant with [^{14}C]G-6-P was minimal (<5%), as verified by using standard [^{14}C]G-6-P.

Quantification of microsomal Ca^{2+} accumulation

Microsomal Ca^{2+} accumulation was evaluated by measuring free Ca^{2+} in incubations with a Ca^{2+} electrode, and was quantified by titrating the amount of Ca^{2+} released by using the Ca^{2+}-ionophore A23187 (2 μM), by means of $CaCl_2$ additions to parallel incubations.

Abbreviations used: G-6-P, glucose 6-phosphate; G-6-Pase, glucose-6-phosphatase.
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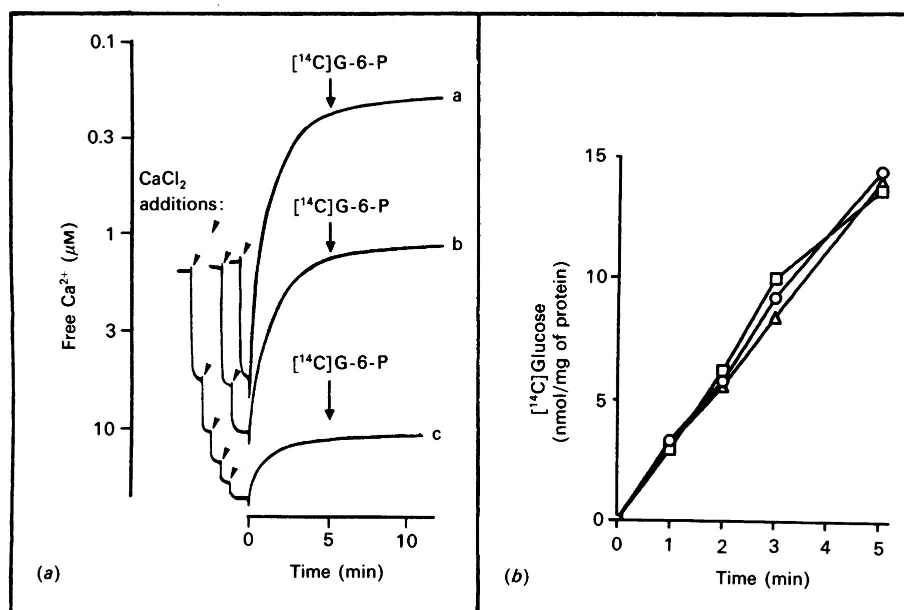

Fig. 1. Buffering at different free Ca^{2+} concentrations of ambient Ca^{2+} (*a*), and concomitant rate of hydrolysis of G-6-P (*b*) by rat liver microsomes

Rat liver microsomes (2 mg of protein/ml) were incubated in the presence of MgATP (see the Experimental section) and different amounts of added Ca^{2+} to obtain different free Ca^{2+} concentrations in the incubation mixture (panel *a*, traces a, b and c). At steady-state levels (arrows), [^{14}C]G-6-P (0.1 μCi/ml, 100 μM) was added to the incubation mixtures. At the indicated time points, labelled glucose was measured as reported in the Experimental section (panel *b*): ○, trace a; □, trace b; △, trace c. Free Ca^{2+} concentration of incubations was measured with a Ca^{2+} electrode. $CaCl_2$ additions were 10 μM each. A typical set of experiments out of four is reported.

Other assays

Protein was determined as reported previously [11], with BSA as standard.

RESULTS AND DISCUSSION

In a first set of experiments, the rate of hydrolysis of G-6-P by intact liver microsomes was evaluated in media containing different concentrations of free Ca^{2+}. This was done by allowing microsomes themselves to buffer external free Ca^{2+} at the desired concentrations. In fact, liver microsomal vesicles possess an active (MgATP-dependent) Ca^{2+}-sequestering system with low capacity (< 10–15 nmol/mg of protein) and high affinity (to 0.1–0.2 μM) [12,13]. In the presence of MgATP and of different amounts of added Ca^{2+} (Fig. 1*a*), liver microsomes accumulated external Ca^{2+} until a steady-state level of approx. 10 nmol/mg of protein was achieved. As a consequence of microsomal sequestration of external Ca^{2+}, desired steady-state levels of free [Ca^{2+}] in the incubation media were obtained (Fig. 1*a*). Under these experimental conditions, the rate of hydrolysis by liver microsomes of added G-6-P (arrows, Fig. 1*a*) was evaluated. As shown in Fig. 1(*b*) and Table 1, liver microsomes linearly hydrolyse 100 μM-G-6-P with a rate which was unaffected by external free Ca^{2+}. Similar results were obtained with 500 μM-G-6-P (Table 1). In order to mimic cytosol-like (physiological) hepatocellular conditions, extravesicular free [Ca^{2+}] from approx. 0.2 to 10 μM and 100 μM- or 500 μM-G-6-P were employed. Indeed, cytosolic free [Ca^{2+}] has been shown to be approx. 0.2 μM in the resting (unstimulated) hepatocytes [12,13], and it rises to 0.5–1 μM after stimulation by extracellular agonists [12,13]. Also, G-6-P concentrations have been reported to be 0.05–0.1 mM in the liver under resting conditions and 0.2–0.5 mM after stimulation (e.g. by hormones) of glycolysis and/or gluconeogenesis [14,15].

Table 1. Rate of hydrolysis of G-6-P by liver microsomes in the presence of different concentrations of ambient free Ca^{2+}

Liver microsomes were incubated in the presence of MgATP and different amounts of Ca^{2+}, and their G-6-Pase activity was subsequently measured with 100 μM- and 500 μM-G-6-P (see Fig. 1 and the Experimental section). Ambient free Ca^{2+} concentrations were measured with a Ca^{2+} electrode. Data are means ± S.E.M. of four experiments.

Free Ca^{2+} concn. (μM)	G-6-P hydrolysis (nmol of glucose/min per mg of protein)	
	100 μM-G-6-P	500 μM-G-6-P
0.22 ± 0.03	2.78 ± 0.61	16.61 ± 2.53
1.3 ± 0.3	2.74 ± 0.42	17.00 ± 2.80
10.9 ± 3.2	2.63 ± 0.45	17.50 ± 1.99

Thus physiological cytosol-like concentrations of extravesicular Ca^{2+} appear unable to modulate the rate of hydrolysis of G-6-P by the liver microsomal G-6-Pase system. This system, however, comprises different components which act in concert in the intact microsomal vesicles [16]. In particular, G-6-P enters the vesicles through a transporter (T1), is hydrolysed intravesicularly by the phosphohydrolase component, and P_i and glucose produced are exported from the vesicles in the medium by two transporters, T2 and T3 respectively [16]. For this, the possibility exists that the rate of hydrolysis of G-6-P would be affected by the level of intravesicular Ca^{2+}, as suggested by others [17]. This possibility, however, is unlikely, since liver microsomes which have released the accumulated Ca^{2+}, after treatment with the Ca^{2+} ionophore A23187 (2 μM; 2 min), exhibit the same G-6-Pase activity (2.61 ± 0.41 nmol of glucose/min per mg of protein; mean ± S.E.M., $n = 3$) as those loaded with Ca^{2+} up to their maximal capacity (2.65 ± 0.53 nmol of glucose/min per mg of protein; mean ± S.E.M., $n = 3$; experimental conditions as in Fig. 1*a*, trace a). After the collapse of the Ca^{2+} gradient by A23187,

Table 2. Rate of hydrolysis of G-6-P by liver microsomes preloaded with different amounts of Ca^{2+} in the presence of P_i

Liver microsomes were incubated in the presence of MgATP, 3 mM-P_i and increasing concentrations of added Ca^{2+} (from 5 to 30 nmol/mg of protein) until free Ca^{2+} concentration of the medium was lowered to approx. 0.2 μM as described in the Experimental section. Microsomal G-6-Pase activity was subsequently measured with 100 μM- or 500 μM-G-6-P, as described in the legend to Fig. 1 and in the Experimental section. Parallel incubations in each experiment were employed to quantify microsomal Ca^{2+} accumulation as described in the Experimental section. Data are means ± S.E.M. of three experiments or means of two experiments.

P_i (mM)	Intravesicular Ca^{2+} (nmol/mg of protein)	G-6-P hydrolysis (nmol of glucose/min per mg of protein)	
		100 μM-G-6-P	500 μM-G-6-P
0	12	2.7	16.0
3	13±3	2.1±0.4	12.9±3.8
3	27±3	2.2±0.3	–
3	40±5	2.0±0.3	13.2±2.0

intravesicular Ca^{2+} concentration should be the same as that of the medium (i.e. 5–6 μM), whereas within Ca^{2+}-loaded vesicles a concentration of approx. 3 mM can be envisaged. The latter value is calculated on the basis of the intravesicular space volume, 6.4±0.7 μl/mg of microsomal protein, reported by others [18].

Previous reports from our laboratory have shown [19,20] that liver microsomes can co-accumulate Ca^{2+} and P_i anions in the presence of MgATP, provided that inorganic phosphates or G-6-P are also present. In the latter case, the hydrolysis of G-6-P by G-6-Pase within the microsomal lumen is the source of accumulated P_i [19]. In the absence of G-6-P, extravesicular inorganic phosphates and Ca^{2+} are co-sequestered by energized microsomes [20]. If similar mechanisms are operative in the intact hepatocyte, high amounts of Ca^{2+} (and P_i) could be stored in the endoplasmic reticulum. On this basis, experiments have been also performed to see if high intramicrosomal levels of Ca^{2+} (and P_i) can affect the rate of hydrolysis of G-6-P by the microsomal G-6-Pase system. As shown in Table 2, in the presence of MgATP and 3 mM-P_i liver microsomes accumulate Ca^{2+} until the external free [Ca^{2+}] is lowered to 0.2 μM. This permits attainment of progressively higher intravesicular Ca^{2+} levels, from 12 to 40 nmol of Ca^{2+}/mg of protein. The rate of G-6-P hydrolysis, however, exhibited by microsomes in all the conditions is unmodified. A small inhibition was present merely as an effect of the inclusion of 3 mM-P_i in the system (Table 2; compare also Table 1).

The reported data compellingly indicate that liver microsomal G-6-Pase activity is not regulated by extravesicular as well as intravesicular Ca^{2+}. At present, although under physiopathological and/or nutritional conditions the amount of G-6-Pase can be increased or decreased [1], no short-term regulation of the enzyme has been definitively proved [16,21].

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