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Calcium sequestration activity in rat liver microsomes. Evidence for a cooperation of calcium transport with glucose-6-phosphatase

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Mechanisms regulating the energy-dependent calcium sequestering activity of liver microsomes were studied. The possibility for a physiologic mechanism capable of entrapping the transported Ca^{2+} was investigated. It was found that the addition of glucose 6-phosphate to the incubation system for MgATP-dependent microsomal calcium transport results in a marked stimulation of Ca^{2+} uptake. The uptake at 30 min is about 50% of that obtained with oxalate when the incubation is carried out at pH 6.8, which is the pH optimum for oxalate-stimulated calcium uptake. However, at physiological pH values (7.2–7.4), the glucose 6-phosphate-stimulated calcium uptake is maximal and equals that obtained with oxalate at pH 6.8. The V_{max} of the glucose 6-phosphate-stimulated transport is 22.3 nmol of calcium/mg protein per min. The apparent K_m for calcium calculated from total calcium concentrations is 31.9 μM. After the incubation of the system for MgATP-dependent microsomal calcium transport in the presence of glucose 6-phosphate, inorganic phosphorus and calcium are found in equal concentrations, on a molar base, in the recovered microsomal fraction. In the system for the glucose 6-phosphate-stimulated calcium uptake, glucose 6-phosphate is actively hydrolyzed by the glucose-6-phosphatase activity of liver microsomes. The latter activity is not influenced by concomitant calcium uptake. Calcium uptake is maximal when the concentration of glucose 6-phosphate in the system is 1–3 mM, which is much lower than that necessary to saturate glucose-6-phosphatase. These results are interpreted in the light of a possible cooperative activity between the energy-dependent calcium pump of liver microsomes and the glucose-6-phosphatase multicomponent system. The physiological implications of such a cooperation are discussed.

Introduction

There is increasing evidence which suggests that calcium plays a central role in regulation of cellular functions, and the mechanisms by which the control of cytoplasmic calcium is achieved in the cell have been extenstively studied. It is generally accepted that calcium levels in the cytoplasm are regulated by at least three processes: one is the modulation of the calcium flux across the plasma membrane, the second is the uptake or release of calcium by or from the mitochondria and the third is the exchange of calcium between cytosol and endoplasmic reticulum.

In the liver cell, an ATP-dependent calcium sequestration activity of the microsomal fraction (vesicles derived principally from the endoplasmic reticulum) was first shown by Moore et al. [1]. The activity was measured [1–4] in the presence of oxalate, which penetrates the microsomal vesicle and precipitates the transported Ca^{2+}. Oxalate was therefore used as a non-physiological entrapping agent which merely served to detect the calcium pumping activity.

A great deal of evidence suggests that in the mitochondria, under physiological conditions, the transported Ca^{2+} interacts with anions such as phosphate to form a non-ionic calcium phosphate complex [5]. Because of such intramitochondrial calcium trapping, extremely large amounts of calcium can be taken up by isolated mitochondria [5,6]. So far no evidence of a similar mechanism has been forwarded for liver microsomes. Therefore, the possibility was investigated that in liver microsomes a physiological system acts in concert with the energy-dependent calcium transport system and generates anions capable of interacting with transported Ca^{2+}. Since it has been shown [7–10] that the active site of glucose-6-phosphatase (EC 3.1.3.9) is located in the luminal surface of the microsomal vesicle, the hydrolysis of glucose 6-phosphate conceivably results in the intravesicular supply of phosphate ions. Such anions could then interact with the actively transported Ca^{2+} and behave as physiological calcium entrapping agents.

The present paper demonstrates that the energy-dependent calcium uptake by liver microsomes is greatly enhanced by the addition of glucose 6-phosphate and provides evidence for a cooperative activity between the ATP-dependent microsomal calcium pump and the glucose-6-phosphatase multicomponent system.

Materials and Methods

Materials

ATP, glucose 6-phosphate, mannose 6-phosphate and bovine serum albumin were obtained from Sigma Chemical Co. St. Louis, MO, U.S.A. $^{45}CaCl_2$ (1650 Ci/mol) was from New England Nuclear, Dreieich, F.R.G. Ruthenium red was from BDH Chemicals, Poole, Dorset, U.K. The ruthenium red was purified by the method of Fletcher et al. [11] and its concentration was determined by measurement of A_{532}, by using an A_{mM} of 61.5 [11]. All the other chemicals were of analytical grade.

Preparation of liver microsomal fraction

Liver microsomal fractions were prepared fresh each day from male Sprague-Dawley rats (230–280 g) maintained on a pellet diet (Nossan, Correzzana, Milan, Italy) and starved overnight before being used. The livers were minced and washed in ice-cold 0.154 M KCl containing 3 mM EDTA at pH 7.4. 10% (w/v) homogenates (2 g of liver were generally used) were prepared in the same medium by four or five passes in a Thomas size-C glass homogenizer fitted with a motor driven (900 rev./min) Teflon pestle (loosely fitting Teflon pestles were used [12]). In any single experiment the homogenate was prepared using the livers derived from three animals. The homogenate was centrifuged at $2700 \times g$ for 10 min. The resulting supernatant fraction was centrifuged at $80000 \times g$ for 30 min. The pellet was resuspended with the homogenization medium to have the microsomal fraction derived from 1 g of liver in 6 ml (protein concentration, 4.5–5.5 mg/ml). The resuspension was made by one or two gentle up-and-down strokes of a hand-held Teflon pestle in a Thomas size-A homogenizer.

The purity of the microsomal fraction, the degree of contamination with mitochondria and the degree of contamination with plasma membranes were checked by testing the activity of glucose-6-phosphatase, cytochrome *c* oxidase [13] and 5′-nucleotidase [13], respectively. It was found that 59.3% of the glucose-6-phosphatase activity present in the whole homogenate was associated with the microsomal fraction, the remainder being associated to the $2700 \times g$ pellet. Also, the microsomal fraction contained 6.2% of cytochrome *c* oxidase activity and 23.9% of the 5′-nucleotidase activity of the whole homogenate. These data are consistent with those reported by Brattin et al. [14] and Reinhart and Bygrave [13] for the assay of the microsomal fraction prepared for studies on calcium transport. Furthermore, the possibility that contaminative mitochondria can account for, at least in part, the energy-dependent Ca^{2+} uptake of the microsomal fraction was vastly reduced by the presence in all the incubation systems of mitochondrial inhibitors (in many experiments sodium azide, 5 mM [1]; in some experiments ruthenium red, 2 μM [3,13], see Table III).

Incubation of liver microsomal fraction

The incubation medium was as follows: 100 mM KCl, 30 mM imidazole histidine buffer (pH 6.8 or 7.2 according to different experiments), 5 mM sodium azide, 5 mM $MgCl_2$, 5 mM ATP, 20

μM $CaCl_2$ and 0.1 μCi/ml $^{45}CaCl_2$. Either 5 mM ammonium oxalate or 10 mM glucose 6-phosphate was added to the medium according to different experiments. The medium was adjusted to the desired pH with imidazole and prewarmed at 37°C for 5 min. The incubation was started by the addition of 0.1 ml of the microsomal suspension (final volume of the sample, 5.0 ml).

In all the experiments, the addition of $CaCl_2$ to the incubation medium was performed by using a stock solution of $CaCl_2$ in 0.3 M HCl. The concentration of $CaCl_2$ in the stock solution (50 mM) was checked by means of atomic-absorption spectroscopy (atomic absorption Spectrophotometer, Perkin Elmer Mod. 5000). In most experiments the Ca^{2+} concentration of the medium was considered to be 20 μM on the basis of added Ca^{2+}, without taking into consideration the amount of calcium present as contaminant of routine solutions. Where indicated, the actual calcium concentration of the medium was measured before and after the addition of $CaCl_2$ by atomic-absorption spectroscopy. The determination was performed after drying the medium at 40°C under house vacuum and addition of 1.8 M HCl to the sample. The amount of calcium present in the medium as contaminant of routine solutions was 3–6 μM.

Calcium uptake assay

Calcium uptake by liver microsomes was measured by using the millipore-filtration technique [1] as follows: at definite time intervals, 500 μl of the incubation mixture were drawn and placed on prewetted cellulose nitrate filters (Sartorius, type SM 113, diameter 25 mm, pore size 0.2 μm). After rapid washing with 10 ml of 100 mM KCl, 30 mM imidazole-histidine buffer (pH 6.8 or 7.2 according to different experiments), the filters were placed in vials and oven-dried. After the addition of 10 ml of scintillation fluid (6 g of 2,5-diphenyloxazone and 50 mg of 1,4-bis(-2-(5-phenyloxazolyl))benzene per liter of toluene) the radioactivity was monitored in a Packard Tri-Carb 3255 scintillation counter. The specific activity of the radioisotope in each incubation mixture was determined by counting an unfiltered 500 μl sample in duplicate after the addition of 10 ml of Instagel (Packard). For each sample the counting efficiency was higher than 95%.

Determination of glucose-6-phosphatase activity

Microsomal glucose-6-phosphatase activity was assayed by measuring the amount of glucose released after the addition of glucose 6-phosphate to the incubation mixture. In fact, due to the presence of ATP in the medium and the Mg^{2+}-Ca^{2+}-dependent ATPases in the microsomal fraction, the glucose-6-phosphatase activity could not be determined by measuring the amount of P_i released from the substrate. In particular, 55 μl of 50% (w/v) trichloroacetic acid were added to 500 μl of the incubation mixture, the sample was centrifuged and glucose was determined in 200 μl of the acid supernatant by the glucose-oxidase method with the kit supplied by Istituto Sieroterapico e Vaccinogeno Toscano 'Sclavo' (Siena, Italy). It was ascertained that the presence of trichloroacetic acid, when used as indicated, did not interfere in the analysis for glucose in the 200 μl aliquot of acid supernatant used for the glucose assay. Also, it was ascertained that in incubates not containing ATP the amount of glucose released from glucose 6-phosphate and detected by the above procedure was equal, on a molar basis, to the amount of released P_i (measured according to Harper [15]).

Determination of P_i and calcium content of microsomal vesicles

The amounts of P_i and calcium accumulated in microsomal vesicles during the incubation with glucose 6-phosphate were determined as follows: 5.0 ml of the incubation mixture (which in these experiments was increased 5-fold with respect to the standard incubations) were drawn and the microsomes were recovered on cellulose nitrate filters (see above). After rapid washing with 60 ml (20 ml, three times) of 100 mM KCl/30 mM imidazole-histidine buffer (pH 7.2) the filter was extracted by shaking with 2 ml of 5% (w/v) trichloroacetic acid for 1 h at room temperature. The acid extract was centrifuged at $9000 \times g$ for 10 min. An aliquot (0.2 ml) of the clear supernatant was used to measure calcium content and another aliquot (0.5 ml) was used to measure P_i content. In some experiments additional aliquots (0.5 ml each) were used to measure glucose (see above) and glucose 6-phosphate content [16].

P_i content of the acid extract was determined

colorimetrically [17] by adding to the samples (i) an equal volume (500 μl) of distilled water, (ii) 50 μl of ammonium molybdate solution ($4 \cdot 10^{-2}$ mol/l, in 20% (v/v) H_2SO_4), (iii) 50 μl of reducing agent [15]. After staying 30 min at room temperature A_{700} was measured. It was previously ascertained, by placing small volumes (10–20 μl) of ATP or glucose 6-phosphate solutions on the filter before the acid extraction and by carrying out the determination as for the experimental samples, that ATP and glucose 6-phosphate are not substantially hydrolyzed during the acid extraction and the colorimetric determination of P_i.

Calcium content was measured by monitoring the radioactivity of the sample (0.2 ml) to which 10 ml of Instagel (Packard) were added. In each experiment more than 92% of the radioactivity present on the filter was recovered in the acid extract.

Other analytical procedures

The amount of P_i released in the incubation medium from the hydrolysis of ATP or glucose 6-phosphate was determined according to Harper [15]. Microsomal protein was determined according to Lowry et al. [18] using bovine serum albumin as a standard.

Results

Fig. 1 shows the energy-dependent calcium uptake by liver microsomes in the presence of either oxalate or glucose 6-phosphate. The experiments were carried out at pH 6.8, which has been reported [1,4] as being the optimal pH for the system containing oxalate as the Ca^{2+}-entrapping agent. As can be seen, calcium uptake in the presence of oxalate was maximal at 30 min and about 10-times greater than in the absence of oxalate (microsomes plus MgATP). Calcium uptake in the presence of glucose 6-phosphate was increased approximately 5-fold as compared to that measured in its absence (microsomes plus MgATP). Virtually no calcium uptake was observed in any of the control incubations carried out in the absence of MgATP (Fig. 1, see legend). Since the addition of glucose 6-phosphate produces an increase of P_i concentration in the incubation system for MgATP-dependent Ca^{2+} uptake (see Fig. 2B), the possibility that such an

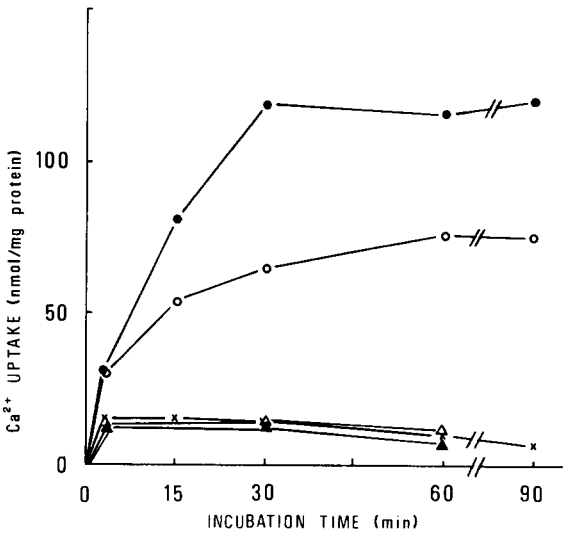

Fig. 1. Time-course of MgATP-dependent Ca^{2+} uptake by liver microsomal fractions in the presence of either oxalate or glucose 6-phosphate at pH 6.8. Liver microsomal fractions were incubated for MgATP-dependent Ca^{2+} uptake as described in Materials and Methods, in the presence of 5 mM ammonium oxalate (●), 10 mM glucose 6-phosphate (○), 1 mM P_i (▲), or 2.5 mM P_i (△), and in the absence of stimulating agents (×). The indicated concentrations of P_i were obtained by adding suitable amounts of K_2HPO_4 to the incubation mixture and the pH value of the medium was adjusted to 6.8. At the indicated time intervals, samples of the incubation mixture were drawn and Ca^{2+} uptake was determined. A typical experiment is reported in the figure. The mean values ± S.E. (six experiments) for Ca^{2+} uptake at 30 min of incubation were 124.7 ± 12.2, 64.6 ± 8.5 and 13.7 ± 1.7 nmol per mg of protein in the presence of 5 mM ammonium oxalate, 10 mM glucose 6-phosphate, and in the absence of stimulating agents, respectively. In the absence of MgATP in the incubation mixture, the mean values ± S.E. for Ca^{2+} uptake at 30 min of incubation were 0.17 ± 0.02, 0.18 ± 0.02 and 0.16 ± 0.03 nmol per mg of protein, in the presence of 5 mM ammonium oxalate, 10 mM glucose 6-phosphate, and in the absence of stimulating agents, respectively.

increase of P_i concentration in the incubation mixture might stimulate microsomal calcium uptake was taken into account. However, as can be seen in Fig. 1, concentrations of P_i similar (1 mM) or higher (2.5 mM) than those measured after 30 min of incubation in the presence of glucose 6-phosphate (see Fig. 2B) did not stimulate microsomal calcium uptake at all.

In the system in which calcium uptake was stimulated by glucose 6-phosphate, the activity of

glucose-6-phosphatase (as measured by the amount of released glucose) was not influenced by concomitant calcium transport (sample in which both MgATP and Ca^{2+} were present) (Fig. 2, panel A). In the same system the hydrolysis of ATP was not influenced by concomitant activity of glucose-6-phosphatase (Fig. 2, panel B). As can be seen, the amount of P_i released in the complete system is practically the sum of the amount released in the system with glucose 6-phosphate (without MgATP and Ca^{2+}) and that released in the system with MgATP and Ca^{2+} (without glucose 6-phosphate). However, from the data shown in Fig. 2, the possibility that glucose 6-phosphate activity has an influence on Ca^{2+}-dependent microsomal ATPases can not be excluded; in fact, the latter activity presumably produces a minor part of the P_i measured in the complete system (i.e., in the presence of MgATP, Ca^{2+} and glucose 6-phosphate), since the Ca^{2+}-dependent ATPases are only part of total microsomal ATPases [1,19] and, furthermore, a large amount of P_i derives from hydrolysis of glucose 6-phosphate.

Since the experiments reported above were carried out at the pH (6.8) optimum for oxalate-stimulated calcium uptake, in further studies the pH optimum for glucose 6-phosphate-stimulated calcium uptake was searched for and the results are given in Fig. 3. As can be seen, the pH (7.2) optimum for glucose 6-phosphate-stimulated calcium uptake was different from the pH (6.8) optimum for oxalate-stimulated calcium uptake. The same amount of calcium was sequestered by

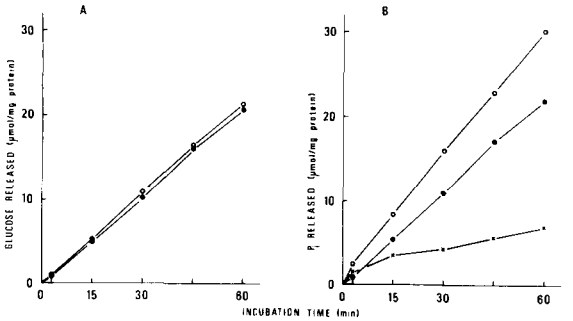

Fig. 2. (A) Time-course release of glucose from glucose 6-phosphate by liver microsomal fraction (glucose-6-phosphatase activity) either in the presence or in the absence of MgATP and Ca^{2+}. Liver microsomal fractions were incubated in the presence of glucose 6-phosphate, as described in Materials and Methods, in the presence (○) or in the absence (●) of MgATP and Ca^{2+}. At the indicated time intervals, samples of the incubation mixture were drawn and glucose was determined. The data reported in the figure are derived from the same experiment as in Fig. 1. The mean values ± S.E. (six experiments) of glucose released at 30 min of incubation were 9.70 ± 0.41 and 10.20 ± 0.32 μmol per mg protein, in the presence and in the absence of MgATP and Ca^{2+}, respectively. (B) Time-course release of P_i from glucose 6-phosphate, from ATP and from both substrates by liver microsomal fraction. Liver microsomal fractions were incubated as described in Materials and Methods, in the presence of glucose 6-phosphate (●), in the presence of MgATP and Ca^{2+} (×), or in the presence of glucose 6-phosphate, MgATP and Ca^{2+} (○). At the indicated time intervals, samples of the incubation mixture were drawn and P_i was determined. Data reported in the figure are derived from the same experiment as in Fig. 1 and in Fig. 2A. The mean values ± S.E. (six experiments) of P_i released at 30 min of incubation were 11.53 ± 0.31, 3.88 ± 0.25 and 15.31 ± 0.33 μmol of P_i per mg of protein in the presence of glucose 6-phosphate, in that of MgATP and Ca^{2+} and in the presence of glucose 6-phosphate, MgATP and Ca^{2+}, respectively.

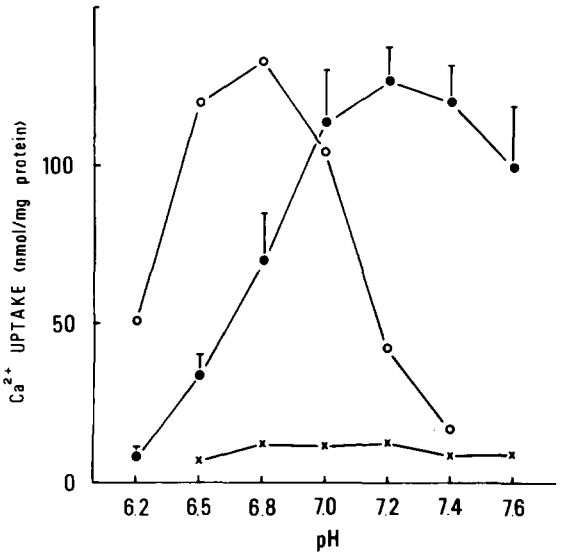

Fig. 3. Effect of pH on MgATP-dependent Ca^{2+} uptake by liver microsomal fraction in the presence of either oxalate or glucose 6-phosphate. Liver microsomal fractions were incubated for MgATP-dependent Ca^{2+} uptake for 30 min. At the end of the incubation, samples of the incubation mixtures were drawn and Ca^{2+} uptake was determined. (○), oxalate (5 mM) -stimulated Ca^{2+} uptake (means of two experiments). (●) Glucose 6-phosphate (10 mM) -stimulated Ca^{2+} uptake (means ± S.E. of three experiments). (×) Ca^{2+} uptake in the absence of stimulating agents (means of two experiments).

liver microsomes in the two systems at the respective optimal pH values. Subsequent studies to further characterize the glucose 6-phosphate-stimulated system were, therefore, carried out at pH 7.2.

Fig. 4A shows the time-course of glucose 6-phosphate-stimulated calcium uptake by liver microsomes. The amount of sequestered calcium at 30 min was 10-times higher than in the absence of glucose 6-phosphate. In the same system, the activity of glucose-6-phosphatase (Fig. 4B) was 24% lower with respect to that measured at pH 6.8 (Fig. 2A). As seen in the experiments carried out at pH 6.8, the activity of glucose-6-phosphatase was not influenced by the concomitant calcium transport (glucose released at 30 min: 8.70 ± 0.46 and 8.40 ± 0.33 μmol/mg protein, in the system containing glucose 6-phosphate only and in that containing glucose 6-phosphate, MgATP and Ca^{2+}, respectively). Similarly, the hydrolysis of ATP was not influenced by concomitant activity of glucose-6-phosphatase (P_i released at 30 min: 8.57 ± 0.57, 2.49 ± 0.11 and 11.37 ± 0.51 μmol/mg protein, in the system containing glucose 6-phosphate, in that containing MgATP and Ca^{2+}, and in the system containing glucose 6-phosphate, MgATP and Ca^{2+}, respectively).

In Fig. 5 the calcium uptake by liver microsomes and the glucose-6-phosphatase activity are plotted against several concentrations of glucose 6-phosphate. While the activity of glucose-6-phosphatase increased linearly with the amount of substrate in the considered range (0.15–10 mM), the calcium uptake was almost maximal at 1.0 mM. At 3.0 mM glucose 6-phosphate concentration (maximal calcium uptake observed), the hydrolysis was nearly 50% of that occurring with 10 mM glucose 6-phosphate. For glucose 6-phosphate concentrations lower than 1 mM there is a linear increase of both glucose-6-phosphatase activity and Ca^{2+} uptake.

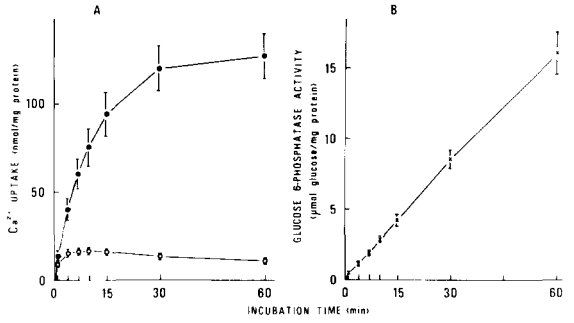

Fig. 4. Time-course of MgATP-dependent, glucose 6-phosphate-stimulated Ca^{2+} uptake by liver microsomal fraction (A) and concomitant glucose-6-phosphatase activity (B) at pH 7.2. Liver microsomal fractions were incubated for MgATP-dependent Ca^{2+} uptake. At the indicated time intervals, samples of the incubation mixture were drawn. Ca^{2+} uptake and the amount of glucose released from glucose 6-phosphate were determined. Results are the mean $\pm$ S.E. of four experiments. ●, Ca^{2+} uptake in the presence of 10 mM glucose 6-phosphate; ○, Ca^{2+} uptake in the absence of glucose 6-phosphate; ×, glucose-6-phosphatase activity. In the absence of MgATP in the incubation mixture, the values for Ca^{2+} uptake at the indicated time intervals were lower than 0.2 nmol of Ca^{2+} per mg of protein both in the presence and in the absence of glucose 6-phosphate.

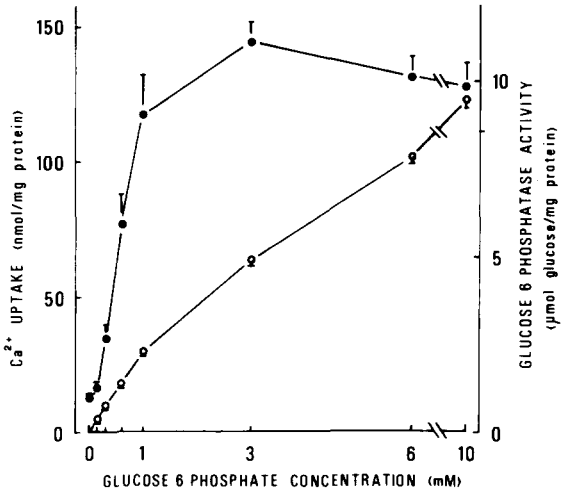

Fig. 5. MgATP-dependent, glucose 6-phosphate-stimulated Ca^{2+} uptake and glucose-6-phosphatase activity of rat liver microsomal fraction as functions of glucose 6-phosphate concentration. Liver microsomal fractions were incubated for MgATP-dependent Ca^{2+} uptake for 30 min, at pH 7.2, in the presence of the indicated concentrations of glucose 6-phosphate. At the end of the incubation, samples of the incubation mixture were drawn. Ca^{2+} uptake and the amount of glucose released from glucose 6-phosphate were determined as described in Materials and Methods. Results are the means $\pm$ S.E. of four experiments. ●, Ca^{2+} uptake; ○, glucose-6-phosphatase activity. The glucose-6-phosphatase activity of liver microsomal fractions incubated in presence of 20 mM glucose 6-phosphate was 10.03 μmol of glucose/mg protein per 30 min. No significant increase in glucose-6-phosphatase activity was observed with glucose 6-phosphate concentrations higher than 20 mM.

The possibility that the enzymatic hydrolysis of glucose 6-phosphate results in the supply of phosphate anions capable of interacting with Ca^{2+} actively transported into the microsomal vesicles was investigated in the experiments reported in Table I. In these experiments, the amount of calcium and P_i was measured in liver microsomes that had been incubated in the system for glucose 6-phosphate-stimulated calcium uptake. As can be seen (Table I), in the absence of glucose 6-phosphate, the amount of calcium taken up by liver microsomes was relatively low and comparable to that shown in Fig. 4A. No attendant accumulation of P_i was found in these microsomes. When, however, glucose 6-phosphate was added to the system, the amount of accumulated calcium was increased about 10-fold (in agreement with that reported in Fig. 4A) and the amount of P_i accumulated in the microsomes at both 15 and 30 min of incubation was virtually equal, on a molar base, to that of the accumulated calcium (Table I). It is important to note that in the samples in which virtually no calcium transport was observed because of the absence of energy supply (MgATP), no accumulation of P_i was found in liver microsomes (Table I) despite attendant hydrolysis of the added glucose 6-phosphate (see legend to Table I; see also Fig. 4B). No significant accumulation of P_i within microsomes during glucose 6-phosphate hydrolysis has also been reported by others [20].

Glucose 6-phosphate is translocated into the microsomal vesicle by the activity of a translocase and then hydrolyzed by the activity of the phosphohydrolase [9,21,22]. The possibility that glucose 6-phosphate interacts itself, within the microsomal vesicle, with the transported calcium seems to be ruled out by the fact that glucose 6-phosphate could not be measured in detectable amounts in the microsomes after the incubation in the system for glucose 6-phosphate-stimulated calcium uptake (see legend to Table I). This would mean that glucose 6-phosphate must be hydrolyzed by

TABLE I

CALCIUM AND P_i CONTENTS OF RAT LIVER MICROSOMAL VESICLES INCUBATED FOR MgATP-DEPENDENT Ca^{2+} UPTAKE STIMULATED BY GLUCOSE 6-PHOSPHATE

Liver microsomal fractions were incubated for MgATP-dependent Ca^{2+} uptake in the presence of the indicated concentrations of glucose 6-phosphate. At the indicated time intervals, samples of the incubation mixture were drawn and were used to measure calcium and P_i content of the microsomal vesicles. P_i values were calculated by subtracting the amount of P_i measured at the start of the incubation (zero time) from that measured at the indicated times. The P_i values (means $\pm$ S.E.) measured at zero time were: 34.6 ± 5.0, 34.3 ± 4.8 and 30.8 ± 3.0 nmol of P_i per mg protein, in the sample containing MgATP and 10 mM glucose 6-phosphate and MgATP, in that containing 10 mM glucose 6-phosphate (no MgATP) and in that containing MgATP (no glucose 6-phosphate), respectively. The amounts of P_i obtained in the absence of both MgATP and glucose 6-phosphate were 31.7 ± 4.3 and 28.5 ± 4.1 nmol of P_i per mg of protein at zero time and at 30 min of incubation, respectively. Calcium content of microsomal vesicles was calculated taking into account the actual calcium content of incubation media (see Materials and Methods). Values reported in the table are the means of five experiments $\pm$ S.E. or the means of two experiments. The glucose-6-phosphatase activity (μmol of glucose per mg of protein) of liver microsomal fractions were: in the presence of 10 mM glucose 6-phosphate 5.40 and 9.05 ± 0.66 at 15 and 30 min of incubation, respectively; in the presence of 10 mM glucose 6-phosphate and MgATP 5.34 and 8.85 ± 0.82, at 15 and 30 min of incubation, respectively; in the presence of 1 mM glucose 6-phosphate 1.98 at 30 min of incubation; in the presence of 1 mM glucose 6-phosphate and MgATP 1.73 at 30 min of incubation. In some experiments, glucose and glucose 6-phosphate were also determined (see Materials and Methods for details). No detectable glucose or glucose 6-phosphate was found in the microsomal fraction in the different incubation systems either at 15 and 30 min of incubation. Results are expressed as nmol per mg protein.

Additions		Incubation time			
MgATP	Glucose 6-phosphate	15 min		30 min	
		Calcium	P_i	Calcium	P_i
+	—	12.8	1.2	10.1 ± 2.1	0
−	10 mM	0.2	0.7	0.2 ± 0.02	0
+	10 mM	95.2	94.3	104.5 ± 10.0	102.1 ± 12.1
−	1 mM	—	—	0.3	—
+	1 mM	—	—	82.6	74.6

the activity of the phosphohydrolase for the entrapping effect on transported calcium to take place.

The possibility that phosphate anions interact with calcium and function as calcium-entrapping agents was further evaluated in experiments in which phosphate anions, at high concentrations, were substituted for glucose 6-phosphate in the incubation system for microsomal calcium uptake. As can be seen in Table II, which shows the calcium uptake at 30 min of incubation, phosphate anions stimulated calcium uptake at concentrations of 5 and 10 mM, but no stimulatory effect was observed with lower concentrations as was observed at pH 6.8 (see Fig. 1). Glucose 6-phosphate, in contrast, stimulated calcium uptake even at a concentration as low as 1 mM.

Contrary to what was observed with glucose 6-phosphate, the effect of phosphate anions on calcium uptake could not be seen at short incubation times (1–3 min; Fig. 6). The lack of a stimulatory effect of P_i on the initial rate of calcium transport has also been reported by others [23]. It is possible that a relatively long period of time is necessary for P_i to reach intravesicular concentrations sufficient to entrap the transported calcium.

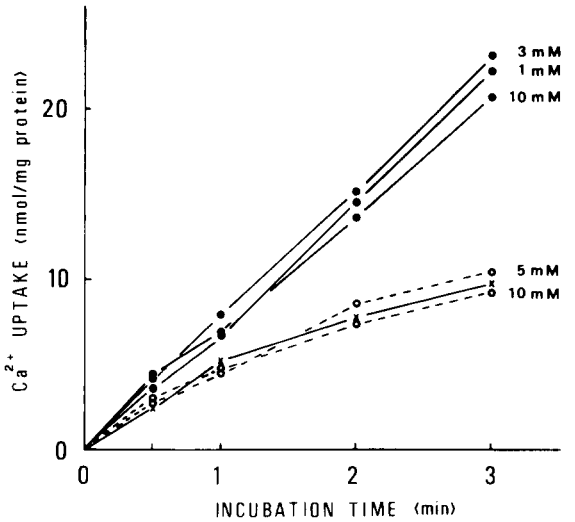

Fig. 6. Effect of glucose 6-phosphate and P_i on the initial rate of MgATP-dependent Ca^{2+} uptake by liver microsomal fraction. Liver microsomal fractions were incubated for MgATP-dependent Ca^{2+} uptake at pH 7.2 as described in Materials and Methods, in the presence of glucose 6-phosphate (●———●) or P_i (○------○) at the concentrations indicated in the figure. Control microsomal fractions were incubated in the absence of glucose 6-phosphate and P_i (×——— ×). At the indicated time intervals, samples of the incubation mixture were drawn and Ca^{2+} uptake was determined as described in Materials and Methods. A typical experiment is reported in the figure.

TABLE II

EFFECT OF P_i ON MgATP-DEPENDENT Ca^{2+} UPTAKE OF LIVER MICROSOMAL FRACTION

Liver microsomal fractions were incubated for MgATP-dependent Ca^{2+} uptake, as described in Materials and Methods, in the presence of glucose 6-phosphate, P_i or mannose 6-phosphate at the indicated concentrations. The indicated concentrations of P_i were obtained by adding suitable amounts of K_2HPO_4 to the incubation mixture, and the pH value of the medium was adjusted to 7.2. At 30 min of incubation, samples of the incubation mixture were drawn and Ca^{2+} uptake was determined. Values are the means ± S.E. of three experiments, or the means of two experiments.

Additions	Ca^{2+} uptake (nmol/mg protein)
None	14.1 ± 1.8
1 mM glucose 6-phosphate	106.9
3 mM glucose 6-phosphate	136.3
10 mM glucose 6-phosphate	121.9
2.5 mM P_i	14.9 ± 2.1
5.0 mM P_i	26.4 ± 1.2
10.0 mM P_i	87.9 ± 9.1
10 mM mannose 6-phosphate	15.3

Substitution of the isomer mannose 6-phosphate for glucose 6-phosphate did not result in any stimulation of calcium uptake (Table II). As is known [24], in fact, mannose 6-phosphate can not be hydrolyzed by intact liver microsomes. In effect, no substantial release of P_i was seen when the microsomal fraction used in the present work was incubated with mannose 6-phosphate in the absence of MgATP and Ca^{2+}. This also means that the microsomal vesicles, as prepared throughout the present study, are characterized by a high level of integrity [25].

The kinetic properties of the microsomal system for MgATP-dependent, glucose 6-phosphate-stimulated calcium uptake were studied. The dependency of the rate of microsomal calcium uptake on the total Ca^{2+} concentration is shown in Fig. 7. With Ca^{2+} concentrations between 0 and 60 μM, there is increasing uptake with increasing Ca^{2+}. From a double-reciprocal plot of the data

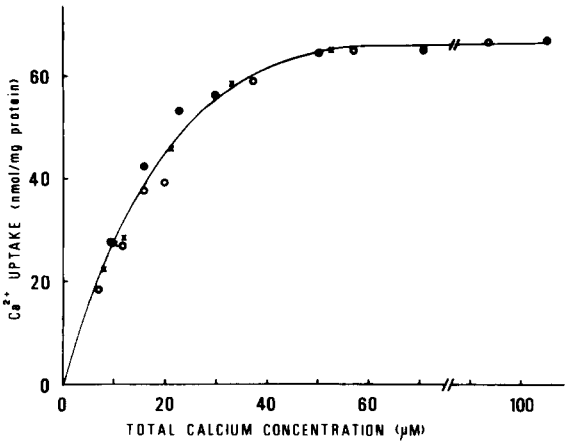

Fig. 7. MgATP-dependent, glucose 6-phosphate-stimulated Ca^{2+} uptake by liver microsomal fraction as a function of total calcium concentration. Liver microsomal fractions were incubated for MgATP-dependent, glucose 6-phosphate-stimulated Ca^{2+} uptake for 5 min, at pH 7.2, as described in Materials and Methods, in the presence of the indicated concentrations of total calcium. The actual concentrations of calcium in the incubation media were determined by atomic absorption spectroscopy. At the end of the incubation, samples of the incubation mixture were drawn and Ca^{2+} uptake was determined. Ca^{2+} uptake values were calculated on the basis of the actual concentrations of total calcium. Data reported in the figure are from three different experiments, indicated by the three different symbols.

TABLE III

MgATP-DEPENDENT, GLUCOSE 6-PHOSPHATE-STIMULATED Ca^{2+} UPTAKE AND GLUCOSE 6-PHOSPHATASE ACTIVITY OF VARIOUS PREPARATIONS OF LIVER MICROSOMAL FRACTIONS

The different liver microsomal fractions were incubated for MgATP-dependent, glucose 6-phosphate-stimulated Ca^{2+} uptake for 30 min at pH 7.2. Results are the mean of two experiments. Liver microsomal fraction was prepared [a] as described in Materials and Methods, sodium azide (5 mM) was present in the incubation medium; [b] as described by Moore et al. [5], sodium azide (5 mM) was present in the incubation medium; [c] from fed rats to obtain the 'heavy microsomes' according to Reinhart and Bygrave [13]. Ruthenium red (2 μM, see Ref. 13) was present in the incubation medium (sodium azide was omitted). Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Microsomal preparation in	Ca^{2+} uptake (nmol Ca^{2+}/ mg of protein)	Glucose-6-phosphatase activity (μmol glucose/mg of protein)
KCl [a]	126.0	9.65
Sucrose [b]	113.8	8.25
Sucrose-Hepes [c]	134.7	8.27

between 0 and 60 μM in Fig. 7, the V_{max} is 22.3 ± 0.6 nmol of calcium/mg of protein per min (mean of three experiments, ± S.E.). The apparent K_m for calcium calculated from the total calcium content of the medium is 31.9 ± 3.0 μM.

Table III shows the glucose 6-phosphate-stimulated calcium uptake and glucose-6-phosphatase activity in the microsomal preparation used in the present work as compared to various microsomal preparations used by other authors [1,13] in studies on calcium transport. As can be seen, the activities for both calcium uptake and glucose-6-phosphatase are comparable in the various microsomal preparations.

Discussion

According to various estimates [26], the free calcium ion concentration in the cytosol of the resting cell is 100–1000-times lower than that occurring in the two subcellular compartments, mitochondria and endoplasmic reticulum. It is generally accepted that the energy-dependent calcium sequestration activity of the endoplasmic reticulum plays a significant role in the maintainance of the cytosolic Ca^{2+} concentration at these relatively low, physiologic levels. However, the physiologic mechanisms by which the endoplasmic reticulum is capable of entrapping the actively transported calcium are not known.

The experimental evidence obtained in the present study demonstrates the following points: (i) the addition of glucose 6-phosphate to the system for energy-dependent microsomal calcium transport results in a marked stimulation of calcium uptake. The uptake dependent on glucose 6-phosphate is about 50% of that obtained with oxalate, when the incubation is carried out at pH 6.8, which is the pH optimum for the oxalate-stimulated calcium uptake. However, at physiological pH values (7.2–7.4), the glucose 6-phosphate-stimulated calcium uptake equals that obtained with oxalate at pH 6.8; (ii) after the incubation of the energized microsomes in the presence of glucose 6-phosphate, P_i and Ca^{2+} are found in equal concentrations, on a molar base, in the microsomal vesicles; (iii) in the system for glucose 6-phosphate-stimulated calcium uptake, glucose 6-phosphate is actively hydrolyzed by the glucose-6-

phosphatase activity of liver microsomes. Such an activity is not influenced by the concomitant calcium uptake; the latter is maximal when the concentration of glucose 6-phosphate in the system is 1–3 mM, which is much lower than that necessary to saturate glucose-6-phosphatase.

These results strongly suggest that the stimulatory effect of glucose 6-phosphate on the energy-dependent microsomal calcium uptake is due to the supply of phosphate ions inside the microsomal vesicle through the glucose-6-phosphatase-mediated hydrolysis of glucose 6-phosphate itself, and that the phosphate ions so supplied act as physiological entrapping agents by precipitating the actively transported calcium.

The hypothesis for a cooperation between active calcium transport and glucose-6-phosphatase activity in the endoplasmic reticulum of the liver cell is strengthened by additional theoretical considerations: (i) the functional significance for the localization of glucose 6-phosphate phosphohydrolase on the luminal surface of the endoplasmic reticulum [20–22] could be explained by the cooperation mentioned above; (ii) the concentrations of glucose 6-phosphate which stimulate calcium uptake are of the same order of magnitude as those (about 0.1 mM) that have been reported [27] to occur in the living liver cell; (iii) the kinetic parameters (V_{max} = 22.3 nmol of Ca^{2+} per min per mg of protein; K_m = 31.9 μM, based on total calcium) of the MgATP-dependent, glucose 6-phosphate-stimulated microsomal Ca^{2+} uptake can account for a buffering capacity of endoplasmic reticulum towards any rise in cytosolic calcium (up to μM concentrations) produced by physiological stimuli [28–30].

It has been shown [13] that the subcellular distribution of ruthenium red-insensitive (non-mitochondrial) calcium transport in adult rat liver follows that of glucose-6-phosphatase. Also, it has been shown [13] that both the activities, which are mainly concentrated in the microsomal fraction, begin to be expressed concomitantly soon after birth. Administration of glucagon to foetuses stimulates ruthenium red-insensitive calcium transport and glucose-6-phosphatase, and maximal stimulation of each activity occurs immediately before birth [13]. These observations reinforce the view that the energy-dependent calcium sequestration activity and glucose-6-phosphatase are physiologically linked in the endoplasmic reticulum of the liver cell and that physiologic events producing changes in the redistribution of free calcium ions in the subcellular compartments may result in activation of both the activities.

The contribution of the calcium pump of the endoplasmic reticulum of the liver cell to the homeostatic mechanisms regulating the concentration of cytosolic Ca^{2+} would have to be considered of secondary importance, at least in comparison to that of the mitochondrial calcium pump, if a mechanism capable of sequestering calcium inside the microsomal vesicles could not be postulated. So far, such a sequestration mechanism has been simulated by the use of oxalate. The possibility that glucose-6-phosphatase provides the mechanism capable of supplying the physiological calcium-entrapping agent makes it reasonable to consider the calcium-sequestering activity of the endoplasmic reticulum of great importance in cellular calcium homeostasis. Glucose-6-phosphatase is, in fact, an enzyme strongly represented in the endoplasmic reticulum and it is probably capable of supplying phosphate anions continuously [20]. Also, it has been demonstrated [31] that, in the absence of Ca^{2+}-entrapping agents, the progressive accumulation of Ca^{2+} inside the microsomal vesicles progressively inhibits the rate of inward Ca^{2+} transport. Thus, any system capable of entrapping Ca^{2+} may decrease the inhibitory activity of the transported Ca^{2+} on Ca^{2+} transport, and may therefore significantly enhance the calcium-sequestering capacity of the endoplasmic reticulum. Studies are in progress to test the possibility that calcium accumulated in the microsomal vesicles under the conditions reported above is released under the stimulation of physiological mediators, such as *myo*-inositol-1,3,5-triphosphate [32], which is known to be the effector of the release of non-mitochondrial calcium into the cytosol [33,34].

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