Effects of Ca^{2+} and V^{5+} on Glucose-6-phosphatase Activity in Rat Liver Microsomes: The Ca^{2+} Effect Is Reversed by Regucalcin

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The effect of regucalcin, a calcium-binding protein isolated from rat liver cytosol, on glucose-6-phosphatase in the microsomes of rat liver was investigated. Addition of Ca^{2+} up to 2.5 μM to the enzyme reaction mixture caused a significant increase of glucose-6-phosphatase activity in hepatic microsomes, while Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+} and Co^{2+} (20 μM) did not have an appreciable effect. Vanadate (V^{5+}) markedly inhibited the enzyme activity; a significant inhibitory effect was seen at 10 μM V^{5+}. The Ca^{2+}-induced increase of glucose-6-phosphatase activity was reversed by the presence of regucalcin; the effect was complete at 1.0 μM of the protein. Regucalcium had no effect on the basal activity of the enzyme. Meanwhile, the inhibitory effect of V^{5+} (10—100 μM) on glucose-6-phosphatase was not appreciably blocked by the presence of regucalcin (up to 2.0 μM). The present data suggest that hepatic microsomal glucose-6-phosphatase is uniquely regulated by Ca^{2+} and V^{5+}, of various metals, and that the Ca^{2+} effect is reversed by regucalcin. The present study supports the view that regucalcin plays an important role as a regulatory protein in liver cell function related to Ca^{2+}.

Keywords regucalcin; calcium-binding protein; calcium; vanadate; glucose-6-phosphatase; rat liver microsome

In recent years, it has been demonstrated that liver metabolism is regulated by increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation.[1,2] The Ca^{2+} effect is amplified through calmodulin, a calcium-binding protein, in liver cells.[3,4] Recently, we have reported that a calcium-binding protein (regucalcin), which differs from calmodulin, is distributed in the hepatic cytosol of rats.[5–7] This novel protein has a reversible effect on the activation of enzyme by Ca^{2+} in liver cells.[6–8] The cell physiological role of regucalcin to regulate liver cell function, however, has not been fully clarified.

On the other hand, glucose-6-phosphatase in the microsomes of rat liver regulates glucose-6-phosphate hydrolysis.[9] Activation of this enzyme by hormone stimulates glucose production from liver cells.[9] The present study was therefore undertaken to investigate the regulatory effect of a calcium-binding protein regucalcin on glucose-6-phosphatase in rat liver microsomes. It was found that, of various metal ions, the enzyme is regulated by Ca^{2+} and vanadate (V^{5+}), and that regucalcin can uniquely reverse the Ca^{2+} effect. The present finding supports the view that regucalcin plays a regulatory role in the stimulation of liver cell function by Ca^{2+}.

Materials and Methods

Animals Male Wistar rats, weighing 100—120 g, were used. They were obtained commercially from Shizuoka Laboratory Animals Center, Hamamatsu, Japan. The animals were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca, and 1.1% P at a room temperature of 25 °C, and were allowed distilled water freely.

Isolation of Regucalcin Regucalcin in the cytosol fraction of rat liver was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously.[10]

Preparation of Hepatic Microsomes Rats were sacrificed by cardiac puncture, and the liver was perfused with ice-cold 0.25 M sucrose solution, frozen immediately, cut into small pieces, suspended 1 : 9 in 0.25 M sucrose solution and homogenized in Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 12000 × *g* in a refrigerated centrifuge for 10 min to remove mitochondria. The 12000 × *g* supernatant was spun at 105000 × *g* for 60 min to obtain the microsomal fraction.[11] The microsomal preparation for enzyme analysis was re-suspended in ice-cold 0.25 M sucrose solution.

Analytical Methods Glucose-6-phosphatase activity was measured by incubation for 20 min at 37 °C in the reaction mixture (final volume, 1.0 ml) containing 40 mM glucose-6-phosphate, 7.0 mM histidine (pH 6.5), various metal ions, regucalcin, and the microsomes (180—200 μg as protein).[12] The amount of inorganic phosphate released by the enzyme reaction was measured according to the method of Nakamura and Mori.[13] In this procedure, a linear relationship exists between time and the amount of protein assayed. The enzyme activity is expressed as nmol of inorganic phosphate released per min per mg protein.

Protein concentration was determined by the method of Lowry *et al.*[14]

Calcium content in the microsomes was determined by atomic absorption spectrophotometry after digestion with nitric acid, and expressed as amount of calcium (nmol) per mg protein of the microsomes.

Reagents All reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and were dissolved in distilled water then passed through an ion-exchange resin to remove metal ions.

Statistical Methods The significance of differences between values was estimated by using Student's *t* test; *p* values of less than 0.05 were considered to indicate statistically significant differences.

Results

The effect of various metal ions on glucose-6-phosphatase activity in the microsomes of rat liver is shown in Fig. 1. The concentration of metal ions used in this experiment was 20 μM in the enzyme reaction mixture. Addition of Ca^{2+} caused a significant increase in glucose-6-phosphatase activity. Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+} and Co^{2+} did not have an appreciable effect on the enzyme activity. Meanwhile, vanadate (V^{5+}) produced a remarkable decrease of glucose-6-phosphatase activity. Of various metal ions, Ca^{2+} and V^{5+} could uniquely regulate glucose-6-phosphatase in rat liver microsomes.

The effect of increasing concentrations of added Ca^{2+} on glucose-6-phosphatase activity in hepatic microsomes is shown in Fig. 2. Addition of 0.1 and 1.0 μM Ca^{2+} had no effect on the enzyme activity. Ca^{2+} up to 2.5 μM caused a significant increase in the enzyme activity. At between 5.0 and 10 μM Ca^{2+}, the elevation of the enzyme activity was maximum. The amount of calcium in heparic microsomes was 4.20 ± 0.10 nmol/mg protein. In the enzyme reaction

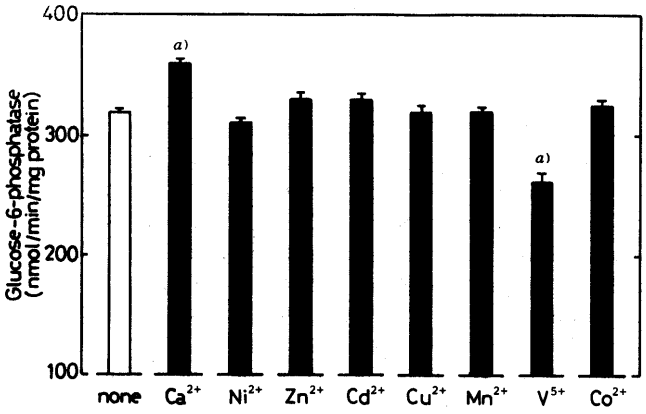

Fig. 1. Effect of Various Metals on Glucose-6-phosphatase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing 20 μM metal as a final concentration. Each value represent the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the control (none) value.

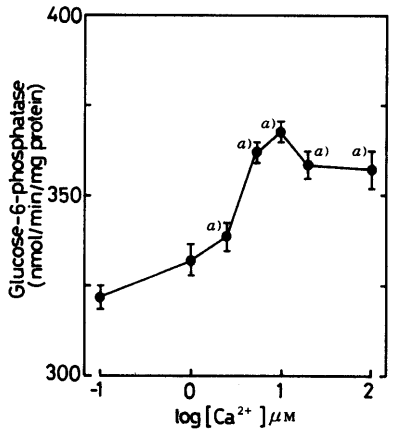

Fig. 2. Effect of Increasing Concentrations of Ca^{2+} on Glucose-6-phosphatase Activity in the Microsomes of Rat Liver

Addition of 10^{-1} μM Ca^{2+} did not affect the enzyme activity. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without Ca^{2+} addition.

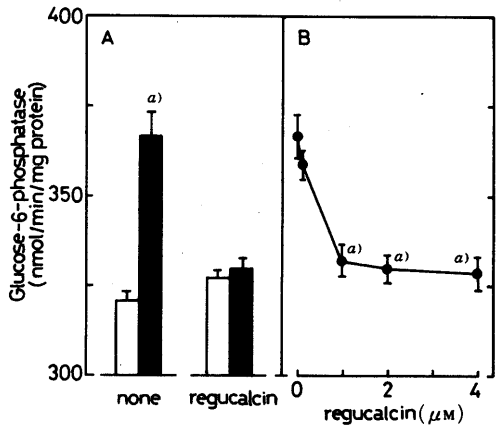

Fig. 3. Effect of Regucalcin on the Ca^{2+}-Induced Increase of Glucose-6-phosphatase Activity in the Microsomes of Rat Liver

Figure A shows the effect of regucalcin (2.0 μM) on the increase of glucose-6-phosphatase activity caused by 10 μM Ca^{2+} addition. Figure B shows the effect of increasing concentrations of regucalcin (0.1, 1.0, 2.0 and 4.0 μM). Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the control value. □, control; ■, 10 μM Ca^{2+} addition.

mixture, microsomal calcium concentration was calculated as 0.76—0.84 μM.

The effect of regucalcin, a calcium-binding protein, on

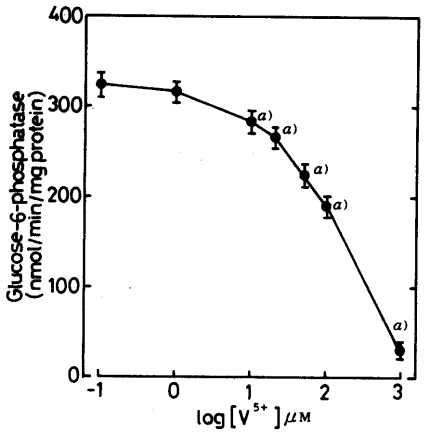

Fig. 4. Effect of Increasing Concentrations of Vanadate (V^{5+}) on Glucose-6-phosphatase Activity in the Microsomes of Rat Liver

Addition of 0.1 or 1.0 μM V^{5+} did not alter the enzyme activity. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without V^{5+}

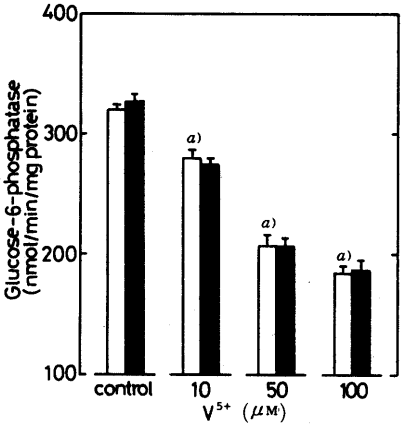

Fig. 5. Effect of Regucalcin on V^{5+}-Induced Inhibition of Glucose-6-phosphatase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing 10, 50 and 100 μM V^{5+} (final concentration) in the presence or absence of 2.0 μM regucalcin. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the control (none) value. □, none; ■, regucalcin.

glucose-6-phosphatase activity in hepatic microsomes is shown in Fig. 3. The presence of regucalcin (2.0 μM) in the enzyme reaction mixture did not have an appreciable effect on the basal activity of glucose-6-phosphatase (Fig. 3A). The increase of the enzyme activity caused by addition of Ca^{2+} (10 μM) was completely reversed by the presence of regucalcin (2.0 μM). At 0.1 μM regucalcin, the protein had no effect (Fig. 3B). The reversible effect of regucalcin was saturated at up to 2.0 μM of the protein.

The effect of vanadate (V^{5+}) at increasing concentrations on glucose-6-phosphatase activity in hepatic microsomes is shown in Fig. 4. Addition of 1.0 μM V^{5+} did not cause a significant decrease in glucose-6-phosphatase activity. At 10 μM V^{5+}, the enzyme activity was decreased significantly. At higher concentrations, the effect was remarkable.

The effect of regucalcin on glucose-6-phosphatase activity decreased by V^{5+} addition is shown in Fig. 5. The presence of regucalcin (2.0 μM) did not reverse the decrease of enzyme activity. At higher concentrations, the reversing effect of regucalcin was not seen (data not shown).

Discussion

Glucose-6-phosphatase in the microsomes of rat liver regulates hydrolysis of glucose-6-phosphate, and the activation of this enzyme by hormonal stimulation increases glucose production from liver cells.[9] Of various metal ions at a comparatively low concentration (20 μM), Ca^{2+} and V^{5+} could influence glucose-6-phosphatase activity in hepatic microsomes, while Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+} and Co^{2+} did not have this effect; the enzyme activity was increased by Ca^{2+} and decreased by V^{5+}. Thus, glucose-6-phosphatase was sensitive to Ca^{2+} and V^{5+}. This finding suggests that Ca^{2+} and V^{5+} may regulate the enzyme in liver cells. At present, the mechanism by which Ca^{2+} and V^{5+} regulate glucose-6-phosphatase activity is unknown. V^{5+} has been found to inhibit several phosphate-hydrolyzing enzymes, including the Na^+- and K^+-stimulated adenosine triphosphatase (ATPase) and alkaline phosphatase.[15-17] V^{5+} may be an essential trace element. The physiological concentration of V^{5+} in liver cells, however, is not known. Since a comparatively low concentration of V^{5+} can inhibit hepatic microsomal glucose-6-phosphatase, the metal may play a regulatory role in glucose metabolism in liver cells.

Ca^{2+} can regulate many enzymes in liver cells, and the metal ion plays an important role as a second messenger of hormonal stimulation.[1,2] Glucose-6-phosphatase in hepatic microsomes was appreciably activated by addition of Ca^{2+} up to 2.5 μM. The amount of endogenous calcium contained in the microsomes used in the enzyme reaction corresponded to approximately 0.8 μM. At Ca^{2+} concentrations higher than the endogenous microsomal calcium level, glucose-6-phosphatase was activated. This activation, however, was not further enhanced by Ca^{2+} addition up to 10 μM. Thus, activation of microsomal glucose-6-phosphatase was saturated in the range of the physiological concentration of Ca^{2+} in liver cells. Ca^{2+} may play a physiological role in the regulation of glucose-6-phosphatase in hepatic microsomes. Since the activation of this enzyme by hormonal stimulation increases glucose production from liver cells,[9] and Ca^{2+} concentration in liver cells is elevated by hormonal stimulation,[1,2] it is possible that the increase in hepatic microsomal glucose-6-phosphatase activity by Ca^{2+} has an appreciable effect in glucose production by liver cells.

Regucalcin is a calcium-binding protein isolated from rat liver cytosol.[5,7] This protein can reverse the activation of hepatic enzymes by Ca^{2+}.[6,8] In the present study, it was found that regucalcin could completely block the increase of glucose-6-phosphatase activity in hepatic microsomes caused by Ca^{2+} addition, though it had no effect on the basal activity of the enzyme. Regucalcin action may be based on Ca^{2+} binding, since the protein has 6—7 high-affinity binding sites per molecule,[5] and the Ca^{2+} binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis.[5]

Glucose-6-phosphatase in hepatic microsomes was markedly inhibited by V^{5+}. This inhibition was not reversed by the presence of regucalcin. The previous investigation showed that 5′-nucleotidase in hepatic plasma membranes was clearly inhibited by 5 μM Zn^{2+}, and that this inhibition was not blocked by the presence of regucalcin.[7] More recently, it was reported that other calcium-binding proteins (calmodulin, S100 b protein and calregulin) can bind zinc.[18,19] If regucalcin could bind V^{5+} and Zn^{2+}, the protein might be expected to reverse the actions of those metals. It seems that regucalcin uniquely reverse the effect of Ca^{2+} among various metal ions.

In conclusion, glucose-6-phosphatase, a rate-limiting enzyme in gluconeogenesis, in the microsomes of rat liver was found to be uniquely regulated by Ca^{2+} and V^{5+}. The effect of Ca^{2+} was completely reversed by the hepatic calcium-binding protein regucalcin. The present study further supports the view that regucalcin plays a role as a regulatory protein in liver cell function related to Ca^{2+}.

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