Activation of Hepatic Microsomal Ca^{2+}-Adenosine Triphosphatase by Calcium-Binding Protein Regucalcin

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The effect of regucalcin, a calcium-binding protein isolated from rat liver cytosol, on Ca^{2+}-adenosine triphosphatase (ATPase) activity in hepatic microsomes was investigated. Mg^{2+}-ATPase activity was clearly increased by the presence of 50 μM Ca^{2+}. Regucalcin (1.0—4.0 μM) caused a remarkable elevation (about 3-fold) of Ca^{2+}-ATPase activity. Also, Mg^{2+}-ATPase activity was increased (about 1.6-fold) by the presence of regucalcin (2.0 and 4.0 μM). Guanosine-5′-*O*-(3-thiotriphosphate) (GTP_rS; 10^{-5} and 10^{-4} M) and nicotinamide adenine dinucleotide phosphate oxidized form ($NADP^+$; 10^{-5} to 10^{-3} M) or reduced form (NADPH; 10^{-4} and 10^{-3} M) significantly increased Ca^{2+}-ATPase activity. These increases were not enhanced by the presence of regucalcin (2.0 μM). Of various metal ions, a comparatively low concentration of V^{5+} (10^{-5} M) or Cd^{2+} (10^{-6} M) significantly increased Ca^{2+}-ATPase activity, while Hg^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+} did not have such an effect. Regucalcin (2.0 μM) did not enhance the effect of V^{5+} and Cd^{2+} on Ca^{2+}-ATPase activity. The present finding, that regucalcin activates hepatic microsomal Ca^{2+}-ATPase, suggests a cell physiological role of regucalcin as an activator in the microsomal Ca^{2+}-pump activity. This action of regucalcin may not be influenced by other regulators.

Keywords regucalcin; calcium-binding protein; Ca^{2+}-ATPase; guanosine-5′-*O*-(3-thiotriphosphate); NADP(H); rat liver microsome

Introduction

It is well known that Ca^{2+} plays an important role in the regulation of many cell functions.[1] The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations.[2,3] More recently, it has been found that a novel calcium-binding protein isolated from rat liver cytosol can reverse the effect of Ca^{2+} on many enzymes in liver cells.[4-8] This protein, which may regulate the Ca^{2+} effects on liver cell function, is called regucalcin.[8] The molecular weight of regucalcin was estimated to be 28800, and the Ca^{2+} binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis.[9] Regucalcin may play a cell physiological role different from that of calmodulin, which can amplify the Ca^{2+} effect,[10] in the regulation of liver cell functions.[4]

On the other hand, the hepatic microsomal fraction, presumably derived from the endoplasmic reticulum, has been demonstrated to possess an adenosine-5′-triphosphate (ATP)-dependent Ca^{2+}-sequestering system.[11,12] This microsomal Ca^{2+}-ATPase is likely to be involved in the regulation of cellular calcium distribution.[13] It has been shown that the microsomal fraction of the liver, together with mitochondria, can lower external free Ca^{2+} concentration to physiological levels.[14] Therefore, the present investigation was undertaken to clarify the effect of regucalcin on Ca^{2+}-ATPase activity in rat liver microsomes. It was found that regucalcin increases hepatic microsomal Ca^{2+}-ATPase activity. This finding suggests that regucalcin plays a role in the regulation of cellular calcium homeostasis.

Materials and Methods

Animals Male Wistar rats, weighing 100—120 g, were purchased 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 and 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.[15,16]

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 a Potter-Elvehjem homogenizer with a Teflon peastle. The homogenate was spun at $12000 \times g$ in a refrigerated centrifuge for 10 min to remove mitochondria. The $12000 \times g$ supernatant was spun at $105000 \times g$ for 60 min to obtain the microsomal fraction. The microsomal preparation for enzyme analysis was re-suspended in ice-cold 0.25 M sucrose solution.

Analytical Methods Mg^{2+}-ATPase activity was determined for 30 min at 25 °C in a medium containing 42.7 mM Hepes-KOH buffer (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 2 mM Tris–ethylene glycol bis(2-aminoethylether)*N,N,N′,N′*-tetraacetic acid (EGTA), 5 mM Tris–ATP and the microsomes (500 μg as protein).[17] The amount of inorganic phosphatase released by the enzyme reaction was measured according to the method of Nakamura and Mori.[18] ($Ca^{2+} + Mg^{2+}$)-ATPase activity was measured in the same medium but with Tris–EGTA replaced by 50 μM $CaCl_2$. Ca^{2+}-ATPase activity was calculated as the difference between ($Ca^{2+} + Mg^{2+}$)-ATPase and Mg^{2+}-ATPase according to Hasselbach and Makinose.[19] The enzyme activity was expressed as nmol of inorganic phosphate released per min per mg protein. Protein concentration was determined by the method of Lowry *et al.*[20]

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 ATP, guanosine-5′-*O*-(3-thiotriphosphate) (GTP_rS) and nicotinamide adenine dinucleotide phosphate oxidized form (β-$NADP^+$) and reduced form (β-NADPH) were purchased form Sigma Chemical Co. (St. Louis, MO, U.S.A.). Vanadium pentoxide and all other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). The reagents were dissolved in distilled water and then passed through ion-exchange resin to remove metal ions.

Statistical Methods The significance of differences between values was estimated by using Student's t-test. A p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

The change of Ca^{2+}-ATPase activity in the microsomes of rat liver as a function of increasing Ca^{2+} concentration is shown in Fig. 1. With higher concentrations than 25 μM Ca^{2+}, Ca^{2+}-ATPase activity increased clearly. The enzyme activity was linearly elevated by the presence of Ca^{2+} in the range of 25—100 μM, but it was not further enhanced by

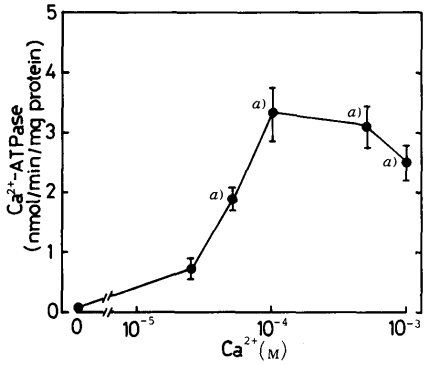

Fig. 1. Change in Ca^{2+}-ATPase Activity with Increasing Concentration of Ca^{2+} in Rat Liver Microsomes

Ca^{2+} was added to the enzyme reaction mixture to give concentrations of 25, 50, 100, 500 and 1000 μM. Each point represents the mean of 5 experiments. The vertical lines give the S.E.M. *a*) $p<0.01$, as compared with the control value.

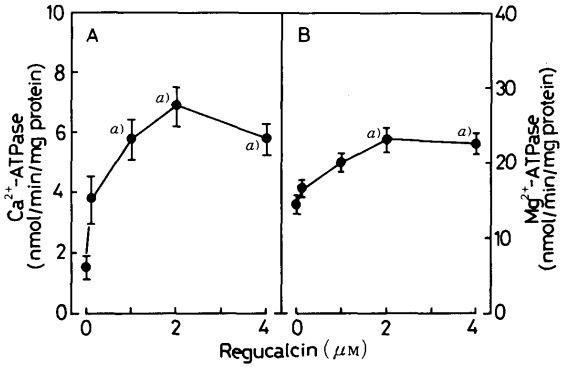

Fig. 2. Effect of Regucalcin on Ca^{2+}-ATPase and Mg^{2+}-ATPase Activities in Rat Liver Microsomes

Regucalcin was added to the enzyme reaction mixture to give concentrations of 0.1, 1.0, 2.0 and 4.0 μM. Each point represents the mean of 5 experiments. The vertical lines give the S.E.M. *a*) $p<0.01$, as compared with the control value.

Ca^{2+} above 100 μM. The amount of calcium which existed endogenously in hepatic microsomes was 4.16 ± 0.15 nmol/mg protein. This amount corresponded to about 2 μM in the enzyme reaction mixture, when it contained 500 μg (as protein) of microsomes.

The effect of regucalcin, a calcium-binding protein isolated from rat liver cytosol, on Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in the hepatic microsomes is shown in Fig. 2. The presence of 1.0 μM regucalcin caused a remarkable elevation of Ca^{2+}-ATPase activity (Fig. 2A). This elevation was about 3-fold over the control value. With higher concentrations of regucalcin, the effect was not further enhanced significantly. The effect of regucalcin on Mg^{2+}-ATPase activity was also examined, and the results is shown in Fig. 2B. The enzyme activity was significantly increased by the presence of 2.0 μM regucalcin, but the effect was not significant at 1.0 μM. This increase was about 1.6-fold over the control value. Further elevation with increasing concentration of regucalcin was not seen. Thus, regucalcin increased both Ca^{2+}-ATPase and Mg^{2+}-ATPase activities. However, regucalcin had a greater effect on Ca^{2+}-ATPase than on Mg^{2+}-ATPase.

To clarify the regulatory mechanism of regucalcin on microsomal Ca^{2+}-ATPase, the effect of GTP_rS on regucalcin-increased Ca^{2+}-ATPase activity in the hepatic

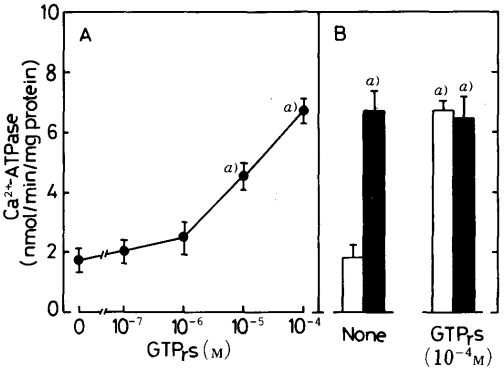

Fig. 3. Effect of GTP_rS on Ca^{2+}-ATPase Activity in Rat Liver Microsomes

Figure A; GTP_rS was added to the enzyme reaction mixture to give concentrations of 10^{-7} to 10^{-4} M. Figure B; both GTP_rS (10^{-4} M) and regucalcin (2.0 μM) were contained in the enzyme reaction mixture. Each point and bar represent the mean of five experiments. The vertical lines give the S.E.M. *a*) $p<0.01$, as compared with the control value. □, control; ■, regucalcin.

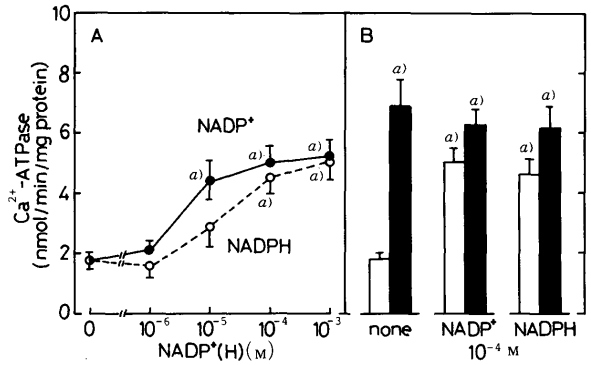

Fig. 4. Effect of $NADP^+$ and NADPH on Ca^{2+}-ATPase Activity in Rat Liver Microsomes

Figure A; $NADP^+$ or NADPH was added to the enzyme reaction mixture to give concentrations of 10^{-6} to 10^{-3} M. Figure B; both $NADP^+$ (10^{-4} M) or NADPH (10^{-4} M) and regucalcin (2.0 μM) were contained in the enzyme reaction mixture. Each point and bar represent the mean of five experiments. The vertical lines give the S.E.M. *a*) $p<0.01$, as compared with the control value. □, control; ■, regucalcin.

microsomes was examined, and the result is shown in Fig. 3. It is known that GTP causes release of Ca^{2+} from rat liver microsomes.[21,22] The presence of GTP_rS (10^{-5} and 10^{-4} M) caused a significant increase in Ca^{2+}-ATPase activity (Fig. 3A). Regucalcin (2.0 μM) did not enhance significantly the effect of GTP_rS to increase Ca^{2+}-ATPase activity (Fig. 3B).

It has been reported that $NADP^+$(H) increases hepatic microsomal ATP-dependent Ca^{2+} pump activity.[23] The effect of $NADP^+$(H) on regucalcin-increased Ca^{2+}-ATPase activity in the hepatic microsomes is shown in Fig. 4. Ca^{2+}-ATPase activity was significantly increased by the presence of $NADP^+$ (Fig. 4A). This increase was seen at 10^{-5} to 10^{-3} M $NADP^+$. Also, NADPH (10^{-4} and 10^{-3} M) increased significantly Ca^{2+}-ATPase activity. The presence of regucalcin (2.0 μM) did not modify the effects of 10^{-4} M $NADP^+$(H) on Ca^{2+}-ATPase activity (Fig. 4B).

The effect of various metal ions on Ca^{2+}-ATPase activity in the hepatic microsomes was examined, and the result is shown in Fig. 5. In the range of 10^{-7} to 10^{-3} M metal ion, addition of Hg^{2+} or Cu^{2+} did not cause an appreciable elevation of Ca^{2+}-ATPase activity. With higher concentrations of Hg^{2+} (10^{-5}—10^{-3} M) and Cu^{2+} (10^{-3} M), Ca^{2+}-

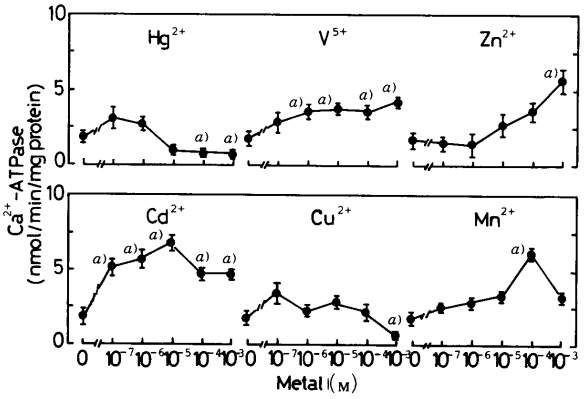

Fig. 5. Effect of Various Metals Ion on Ca^{2+}-ATPase Activity in Rat Liver Microsomes

Various metal ions were added to the enzyme reaction mixture to give concentrations of 10^{-7} to 10^{-3} M. Metals were the chlorides, except for V^{5+} (vanadium pentoxide). Each point represents the mean of four experiments. Vertical lines give the S.E.M. *a*) $p<0.01$, as compared with the control value.

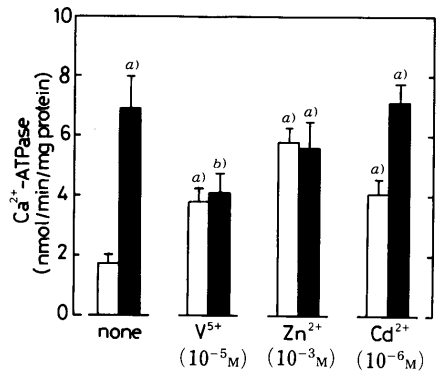

Fig. 6. Effect of Regucalcin on the Increase in Metal-Induced Ca^{2+}-ATPase Activity in Rat Liver Microsomes

Both regucalcin (2.0 μM) and V^{5+} (10^{-5} M), Zn^{2+} (10^{-3} M) or Cd^{2+} (10^{-6} M) were added to the enzyme reaction mixture. Each bar represents the mean of five experiments. Vertical lines give the S.E.M. *a*) $p<0.01$, as compared with the control value. *b*) $p<0.01$, as compared with regucalcin alone. □, control; ■, regucalcin.

ATPase activity was reduced. V^{5+} up to 10^{-6} M significantly increased Ca^{2+}-ATPase activity. Cd^{2+} (10^{-7} to 10^{-3} M) markedly raised Ca^{2+}-ATPase activity. Addition of 10^{-3} M Zn^{2+} caused a significant increase in Ca^{2+}-ATPase activity. With 10^{-4} M Mn^{2+}, the enzyme activity was raised significantly. Thus, Ca^{2+}-ATPase activity was affected by various metal ions at various concentrations. In particular, Cd^{2+} had a considerable effect on hepatic microsomal Ca^{2+}-ATPase among the various metal ions. Next, the effect of regucalcin on the increase in V^{5+}, Zn^{2+} and Cd^{2+}-induced Ca^{2+}-ATPase activity was examined, and the result is shown in Fig. 6. The effect of regucalcin (2.0 μM) to increase Ca^{2+}-ATPase activity was significantly prevented by the presence of 10^{-5} M V^{5+}, although the metal increased the enzyme activity. The present of 10^{-3} M Zn^{2+} or 10^{-6} M Cd^{2+} did not modify the effect of regucalcin on Ca^{2+}-ATPase activity.

Discussion

The ATP-dependent accumulation of Ca^{2+} by the rat liver microsomal fraction is now well documented. It is generally accepted that the enzymatic basis for the ATP-dependent Ca^{2+} accumulation is the microsomal Ca^{2+}-ATPase.[24] In the present study, it has been demonstrated that regucalcin, a calcium-binding protein isolated from rat liver cytosol, can increase Ca^{2+}-ATPase activity in the hepatic microsomes. This increase was about 3-fold over the control. Meanwhile, Mg^{2+}-ATPase activity was also elevated by regucalcin; the elevation was approximately 1.6-fold over the control. Thus, regucalcin had a considerable effect on Ca^{2+}-ATPase rather than Mg^{2+}-ATPase. Iodinated regucalcin could bind to microsomes prepared from rat liver.[25] Regucalcin may directly activate Ca^{2+}-ATPase by binding to the microsomes. Presumably, regucalcin may play a role as an activator of hepatic microsomal Ca^{2+}-ATPase. This is a novel finding concerning the microsomal Ca^{2+}-ATPase activator protein.

It has been reported that GTP (10^{-5} M) greatly enhances the inositol 1,4,5-triphosphate-stimulated Ca^{2+} release from rat liver microsomes.[21,22] In our investigation, the hepatic microsomal Ca^{2+}-ATPase activity was significantly raised by addition of GTP_rs (10^{-5} and 10^{-4} M). This result raises the possibility that GTP can stimulate Ca^{2+} uptake by the microsomes, although GTP causes the release of Ca^{2+} from rat liver microsomes.[21,22] In the microsomal Ca^{2+} transport system, GTP may regulate both uptake and release of Ca^{2+}. Regucalcin did not enhance significantly GTP_rs (10^{-4} M)-increased Ca^{2+}-ATPase activity in the hepatic microsomes. This suggests that regucalcin influences the same site as that for GTP action on hepatic microsomal Ca^{2+}-ATPase.

There is growing evidence that a cytochrome P-450 and a thiol-protein disulfide oxidoreductase may directly regulate the microsomal ATP-dependent Ca^{2+} pump activity.[23,26] Recently, it has been reported that $NADP^+(H)$ (10^{-4} M) can stimulate the uptake of Ca^{2+} by the hepatic microsomes.[26] In the present study, it has been demonstrated that the microsomal Ca^{2+}-ATPase activity is increased by $NADP^+$ (10^{-5} to 10^{-3} M) and NADPH (10^{-4} and 10^{-3} M). Regucalcin did not cause a significant alteration of $NADP^+(H)$-increased Ca^{2+}-ATPase activity. Presumably, the site of regucalcin action on hepatic microsomal Ca^{2+}-ATPase is involved in the regulatory site of $NADP^+(H)$.

Interestingly, the hepatic microsomal Ca^{2+}-ATPase activity was significantly increased by the presence of V^{5+} (10^{-6} to 10^{-3} M), Cd^{2+} (10^{-7} to 10^{-3} M), Zn^{2+} (10^{-3} M) and Mn^{2+} (10^{-4} M),while Hg^{2+} and Cu^{2+} could not elevate the enzyme activity. Of various metal ions, V^{5+} and Cd^{2+} could uniquely activate Ca^{2+}-ATPase. We do not yet know the physiological significance of Ca^{2+}-ATPase activation by V^{5+} and Cd^{2+}. It is possible that low concentrations of V^{5+} and Cd^{2+} may regulate hepatic microsomal Ca^{2+}-ATPase. The increase in the microsomal Ca^{2+}-ATPase activity induced by V^{5+} and Cd^{2+} was not altered significantly by the presence of regucalcin. In the presence of V^{5+} (10^{-5} M), however, the regucalcin effect on Ca^{2+}-ATPase was partly inhibited. Regucalcin did not bind V^{5+} (data not shown), but the metal may influence the binding of regucalcin to the microsomes. Presumably, calcium-binding protein regucalcin can not bind metals other than Ca^{2+}.

The present investigation demonstrates that the hepatic microsomal Ca^{2+}-ATPase activity is regulated by many factors: regucalcin, GTP_rs, $NADP^+(H)$ and metals (especially V^{5+} and Cd^{2+}). It is not certain whether the regulatory sites of those factors on hepatic microsomal

Ca^{2+}-ATPase are identical. Regucalcin did not modify the effects of other regulators. The action of regucalcin to activate Ca^{2+}-ATPase may not be related to a specific regulatory site. The mechanism of regucalcin action remains to be elucidated.

In conclusion, it has been demonstrated that regucalcin, a calcium-binding protein isolated from rat liver cytosol, can activate Ca^{2+}-ATPase in hepatic microsomes. The present finding suggests that regucalcin plays a cell physiological role related to hepatic microsomal Ca^{2+} pump activity in the regulation of intracellular Ca^{2+} homeostasis.

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