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Calcium-Binding Protein Regucalcin Is an Activator of (Ca^{2+}–Mg^{2+})-Adenosine Triphosphatase in the Plasma Membranes of Rat Liver

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The effect of regucalcin, a calcium-binding protein isolated from rat liver cytosol, on (Ca^{2+}–Mg^{2+})-adenosine triphosphatase (ATPase) activity in the plasma membranes of rat liver was investigated. ^{125}I-Regucalcin bound to the plasma membranes in the presence or absence of 0.1 mM Ca^{2+}. Regucalcin (0.35 μM) increased the plasma membrane (Ca^{2+}–Mg^{2+})-ATPase activity about 15% ($p<0.01$), and a higher concentration (4.0 μM) showed a remarkable effect. Also, regucalcin (0.35—2.0 μM) markedly increased the plasma membrane Mg^{2+}-ATPase activity in the absence of Ca^{2+}. The effect of regucalcin on (Ca^{2+}–Mg^{2+})-ATPase activity was not regulated by the presence of guanosine-5′-*O*-(3-thiotriphosphate) (10^{-5} and 10^{-4} M), glucagon (10^{-6} and 10^{-5} M) or norepinephrine (10^{-7}—10^{-5} M), suggesting that the regucalcin effect is not linked to guanosine triphosphate (GTP)-binding protein in the plasma membranes. Vanadium (10 and 10 μM), which inhibits the phosphorylation of liver plasma membrane (Ca^{2+}–Mg^{2+})-ATPase, decreased the enzyme activity about 20% ($p<0.01$). This decrease was completely restored by the presence of regucalcin (1.0 μM). The present results indicate that regucalcin binds to the plasma membranes of liver cells and increases the (Ca^{2+}–Mg^{2+})-ATPase activity. Regucalcin may be an activator of (Ca^{2+}–Mg^{2+})-ATPase in the plasma membranes.

Keywords——regucalcin; calcium-binding protein; calcium; (Ca^{2+}–Mg^{2+})-ATPase; rat liver plasma membrane

Introduction

It is well known that Ca^{2+} plays an important role in the regulation of many cell functions.[1,2] The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations.[3,4] Liver metabolism is regulated by increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation.[3,4] Calmodulin, a calcium-binding protein, can amplify the metabolic effect of the cytosolic Ca^{2+} in liver cells; a function of calmodulin is activation of many enzymes.[1,2] Recently, we have reported that a calcium-binding protein, which differs from calmodulin, is distributed in the hepatic cytosol of rats.[5–8] This novel protein has a reversible effect on both activation and inhibition of enzymes by Ca^{2+} in liver cells.[9–13] We therefore proposed that this calcium-binding protein should be called regucalcin.[13] This protein probably plays an important role in the regulation of liver cell functions related to Ca^{2+}.

In liver, the mechanism of Ca^{2+} extrusion is poorly understood. More recently, it has been demonstrated that the high-affinity (Ca^{2+}–Mg^{2+})-adenosine triphosphatase (ATPase) is located on the plasma membranes of rat liver,[14–18] and that this enzyme acts as a Ca^{2+} pump to extrude the metal ion from the cytosol of liver cells.[18] Therefore, the present investigation

was undertaken to clarify whether regucalcin can regulate (Ca^{2+}–Mg^{2+})-ATPase activity in the plasma membranes of rat liver. It was found that regucalcin binds to the plasma membranes of liver cells and increases the plasma membranous (Ca^{2+}–Mg^{2+})-ATPase activity. The present results support the view that regucalcin is an activator of (Ca^{2+}–Mg^{2+})-ATPase in the plasma membranes of rat liver.

Materials and Methods

Animals——Male Wistar rats, weighing 100—120 g, were used. They were obtained commercially (Shizuoka Laboratory Animal Center, Hamamatsu, Japan). The animals were given commercial laboratory chow containing 1.1% Ca, 1.1% P and 57.4% carbohydrate (Oriental Test Diet, Tokyo, Japan) and tap water freely.

Reagents——Guanosine-5′-*O*-(3-thiotriphosphate) (GTP_rs), glucagon and norepinephrine were purchased from 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.

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.[6]

Preparation of ^{125}I-Regucalcin——Regucalcin was labelled with $Na^{125}I$ by the method of Hunter and Greenwood,[19] and purified by cellulose acetate electrophoresis. Specific activity achieved was 2.25×10^6 cpm/μg of protein. $Na^{125}I$ (specific activity 17 Ci/mg) used was obtained from New England Nuclear (Boston, Mass., U.S.A.).

Preparation of Liver Subcellular Fraction——The livers were perfused with ice-cold 0.25 M sucrose solution, cut into small pieces, suspended 1 : 9 in 0.25 M sucrose solution and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was laid on ice-cold 0.34 M sucrose solution in a centrifuge tube, and spun at $700 \times g$ in a refrigerated centrifuge for 10 min and the precipitate (nuclei fraction) was collected.[20] Also, the homogenate was spun at $1100 \times g$ in a refrigerated centrifuge for 10 min and the supernatant was spun at $10800 \times g$ (ppt; mitochondrial fraction) for 10 min, and the $10800 \times g$ supernatant was spun at $105000 \times g$ for 60 min. The precipitate (microsomal fraction) and the supernatant (cytosol fraction) were separated. The protein concentration was determined by the method of Lowry *et al.*[21]

Preparation of Liver Plasma Membranes——The liver was removed after perfusion with ice-cold 0.25 M sucrose solution. The liver plasma membranes were prepared according to the procedure of Song *et al.*[22] The livers from three rats were minced and placed in Dounce homogenizers with loose-fitting pestles. Two volumes of 1.0 mM $NaHCO_3$ solution (pH 7.5) were added to approximately 10 g of minced liver in each homogenizer, and the mixture was gently homogenized by 30 strokes of the pestle. The homogenate was diluted with 250 ml of the same solution, passed twice through four layers of surgical gauze, and then centrifuged for 10 min at $1500 \times g$. The pellets were pooled and the suspension of crude membranes was made up to exactly 5.5 volumes of density 1.26 sucrose (70.74%, w/v). Aliquots (13—14 ml) of the suspension were placed in cellulose nitrate tubes, and 7 and 5 ml of sucrose solutions of densities 1.18 (A; 48.45%, w/v) and 1.16 (B; 42.9%, w/v) were layered in succession. The tubes were centrifuged in a Spinco No. 30 rotor for 60 min at $66000 \times g$. The plasma membrane fraction accumulated at the two sucrose interfaces (A and B). Materials from both interfaces were assayed for 5′-nucleotidase, a marker enzyme for plasma membrane.[23] A greater specific activity was found in fractions collected from the upper (B) interface, and contamination by mitochondria and microsomes was slight as estimated from the results of assays for succinate dehydrogenase[24] and glucose-6-phosphatase,[25] respectively. Materials from the upper (B) interface in each tube were collected by means of a syringe, mixed with 20 volumes of 1.0 mM $NaHCO_3$, and centrifuged for 30 min at $10500 \times g$. The resulting pellet was washed once by gentle suspension in about 20 volumes of 1.0 mM $NaHCO_3$ and recentrifuged for 30 min at $10500 \times g$. The plasma membranes were washed, and suspended in ice-cold distilled water. The protein concentration was determined by the method of Lowry *et al.*[21]

Binding Assays of ^{125}I-Regucalcin——Labelled regucalcin was diluted with unlabelled regucalcin to give a final stock solution that contained 0.29 mg/ml regucalcin and about 5000 cpm/μl. Assay buffer consisted of 10 mM Tris–HCl (pH 7.3), 150 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, ^{125}I-regucalcin (0.17—1.4 nM), unlabelled regucalcin (1.0 μM) and the plasma membranes (40 μg as protein) or the subcellular fraction (40 μg as protein), with or without 1.0 mM ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in a final volume of 1.0 ml. After a 30 min incubation at 20°C, 0.5 ml aliquots of the assay sample were taken from the assay tube, and added to microcentrifuge tubes containing assay buffer (0.8 ml). In the case of experiments with the cytosol fraction, the assay buffer contained 5.0% trichloroacetic acid. Labelled regucalcin-bound materials were collected by centrifugation at $10000 \times g$ for 2.0 min. The materials precipitated were washed with assay buffer. The amount of ^{125}I was determined with a gamma counter. The amount of ^{125}I-regucalcin bound was calculated by subtracting values obtained without the subcellular fractions from those obtained with the subcellular fractions. Results were expressed as ng of labelled

regucalcin per mg of protein.

Assay of Liver Plasma Membrane (Ca^{2+}–Mg^{2+})-ATPase——(Ca^{2+}–Mg^{2+})-ATPase in the plasma membranes was measured under conditions described by Lotersztajn *et al.*,[14] except that phosphate release was determined as described elsewhere.[26] The standard assay for (Ca^{2+}–Mg^{2+})-ATPase activity contained in a final volume of 250 μl, 60—65 μg of plasma membranes protein, 250 μM Mg-adenosine triphosphate (ATP), 50 mM Tris–HCl (pH 8.0), 0—4 μM regucalcin, and 400 μM EGTA with either no Ca^{2+} or 400 μM total Ca^{2+} (this corresponds to a free Ca^{2+} concentration of 1.76 μM). The value calculated for total Ca^{2+} concentration takes into account the Ca^{2+} present in the medium and in the plasma membranes added to the assay medium. This concentration was measured by atomic absorption spectroscopy and usually amounted to about 2 μM. After 10 min at 30°C, aliquots were assayed for inorganic phosphate formed. (Ca^{2+}–Mg^{2+})-ATPase activity was calculated by subtracting values obtained with chelator alone from those obtained with chelator plus Ca^{2+}. Results were expressed as nmoles of inorganic phosphate liberated per min per mg of protein.

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

Results

Binding of Radioiodinated Regucalcin to Liver Plasma Membranes

The binding of ^{125}I-regucalcin to the subcellular fractions obtained from rat liver is shown in Fig. 1. Radioiodinated regucalcin bound to the plasma membranes, nuclei, mitochondria and microsomes in the presence of 0.1 mM Ca^{2+}. The binding was weakened, though not significantly, by the presence of 1.0 mM EGTA. Radioiodinated regucalcin also bound to the proteins in the cytosol, when the binding was assayed by precipitation with 5% trichloroacetic acid. The binding of ^{125}I-regucalcin to the subcellular fractions was detected at very low concentrations of the labelled protein. Regucalcin-binding sites in each subcellular fraction were saturated at approximately 40 ng/ml (1.4 nM). However, the binding of ^{125}I-regucalcin was remarkable in the plasma membranes and cytosol, as compared with those of other fractions.

The time-course of binding of ^{125}I-regucalcin to the plasma membranes of rat liver in the presence of 0.1 mM Ca^{2+} is shown in Fig. 2. The binding of ^{125}I regucalcin was apparent within 30 s, became maximal in 5 min, and then began to decrease. An apparent equilibrium of

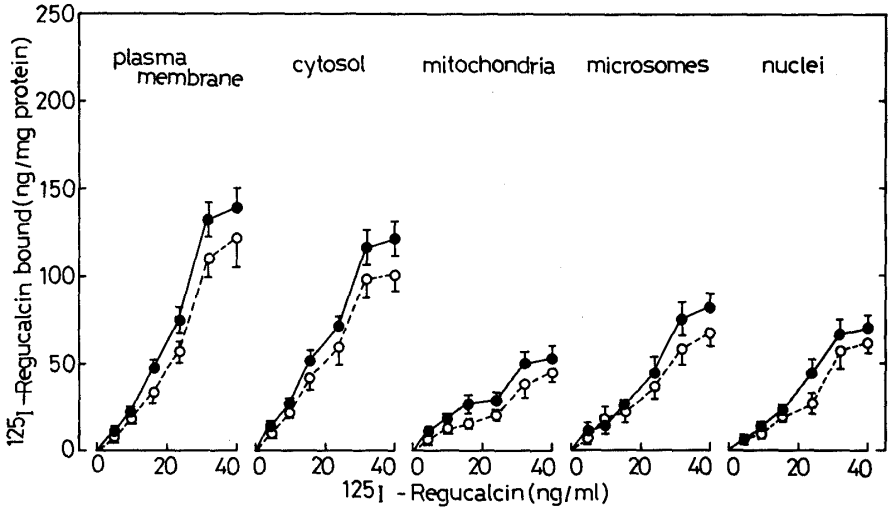

Fig. 1. Binding of Radioiodinated Regucalcin to the Subcellular Fractions of Rat Liver

^{125}I-Regucalcin was incubated with the subcellular fractions (40 μg of protein/ml) for 30 min at 20 °C with various the amount of ^{125}I-regucalcin (4.5—40 ng/ml; 0.17—1.4 nM), as indicated on the abscissa, in the presence of unlabelled regucalcin (29 μg/ml; 1.0 μM). The binding was expressed as ng of ^{125}I-regucalcin bound per mg of the subcellular proteins. Each value represents the mean ± S.E.M. of 5 experiments. ---○---, with 1.0 mM EGTA; —●—, in the presence of 0.1 mM Ca^{2+}.

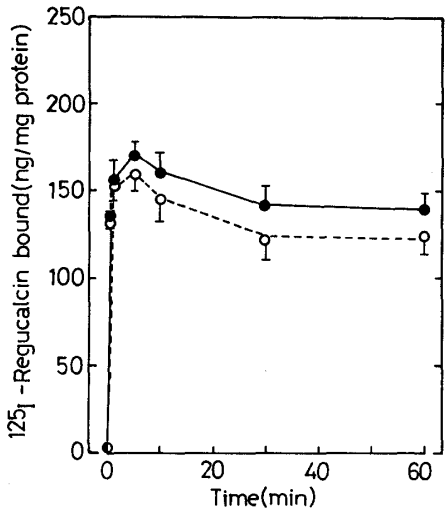

Fig. 2. Time-Course of Binding of ^{125}I-Regucalcin to the Plasma Membranes of Rat Liver

^{125}I-Regucalcin (40 ng/ml; 1.4 nM) was added to the plasma membranes (40 μg/ml) with excess unlabelled regucalcin (29 μg/ml; 1.0 μM). Incubations were carried out at 20 °C. The binding was expressed as ng of radioiodinated regucalcin bound per mg of the plasma membrane protein. Each value represents the mean ± S.E.M. of 5 experiments. ---○---, with 1.0 mM EGTA; —●—, in the presence of 0.1 mM Ca^{2+}.

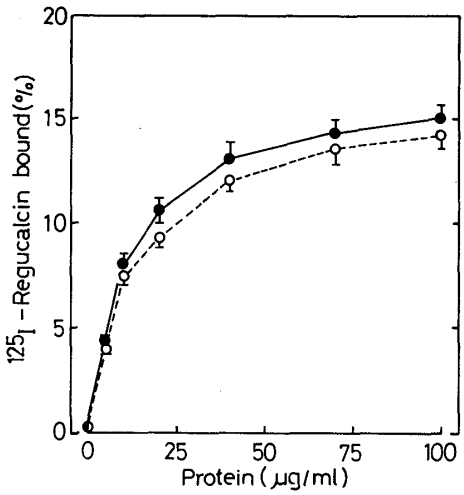

Fig. 3. Binding of [^{125}I]Regucalcin to the Plasma Membranes of Rat Liver as a Function of Protein Concentration

Incubations were carried out for 30 min at 20 °C with at various the plasma membrane concentrations. Each value represents the mean ± S.E.M. of 5 experiments. ---○---, with 1.0 mM EGTA; —●—, in the presence of 0.1 mM Ca^{2+}.

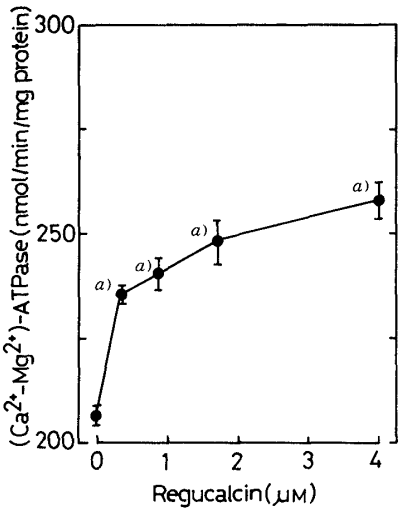

Fig. 4. Effect of Regucalcin on (Ca^{2+}–Mg^{2+})-ATPase Activity in the Plasma Membranes of Rat Liver

The enzyme activity was measured in the reaction mixture containing regucalcin (0.35—4.0 μM). Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p < 0.01$, as compared with the control (none) value.

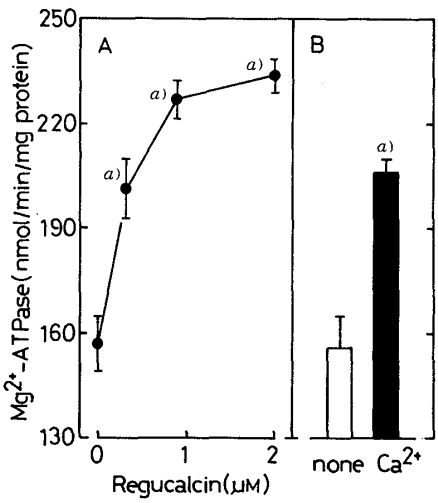

Fig. 5. Effect of Regucalcin on Mg^{2+}-ATPase Activity in the Plasma Membranes of Rat Liver

Figure A shows the effect of increasing concentrations of regucalcin on Mg^{2+}-ATPase activity; the enzyme reaction mixture did not contain Ca^{2+}. Figure B shows the effect of 0.4 mM Ca^{2+} addition on the Mg^{2+}-ATPase activity. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p < 0.01$, as compared with the control (none) value.

^{125}I-regucalcin binding was seen at 30 min and was maintained for 60 min. The binding was slightly decreased by the presence of 1.0 mM EGTA.

When the binding experiments were performed with a 30-min incubation, the binding of ^{125}I-regucalcin was a linear function of the plasma membrane protein concentration up to

10 μg/ml, continued to increase up to 40 μg/ml and was saturated at 70 μg/ml (Fig. 3). At the protein concentration of the plasma membranes of 40 μg/ml, the plasma membranes bound about 13% of added ^{125}I-regucalcin in the presence of unlabelled regucalcin (1.0 μM). This corresponded to 4.86 pmol of radioiodinated regucalcin per mg of the plasma membrane protein.

Effect of Regucalcin on (Ca^{2+}–Mg^{2+})-ATPase Activity in Liver Plasma Membranes

The effect of regucalcin on (Ca^{2+}–Mg^{2+})-ATPase activity in the plasma membranes of rat liver is shown in Fig. 4. The enzyme activity was significantly increased by the presence of 0.35 μM regucalcin. With increasing concentrations (0.85, 1.70 and 4.0 μM), the effect was greater. At 4.0 μM, the enzyme activity was increased about 25% ($p=0.01$) from the control value.

Whether the effect of regucalcin on (Ca^{2+}–Mg^{2+})-ATPase activity is related to the alteration of Ca^{2+} was next examined, because regucalcin can bind Ca^{2+}.[7,8] When the enzyme reaction mixture did not contain Ca^{2+}, the enzyme activity was reduced markedly. In this case, however, the presence of 0.35 μM regucalcin caused a complete restoration of the reduced activity (Fig. 5). Thus, the effect of regucalcin on (Ca^{2+}–Mg^{2+})-ATPase did not result from the alteration of Ca^{2+}.

Effect of GTP_rS and Hormones on Regucalcin-Increased (Ca^{2+}–Mg^{2+})-ATPase Activity in Liver Plasma Membranes

The effect of GTP_rS and glucagon on (Ca^{2+}–Mg^{2+})-ATPase activity in the plasma membranes of rat liver is shown in Fig. 6. GTP_rS (10^{-5} and 10^{-4} M) did not significantly change the enzyme activity of the control. Also, GTP_rS did not have a significant effect on regucalcin (1.0 μM)-increased enzyme activity. Glucagon (10^{-6} and 10^{-5} M) significantly increased (Ca^{2+}–Mg^{2+})-ATPase activity. However, the hormone did not influence regucalcin (1.0 μM)-increased enzyme activity. Norepinephrine (10^{-7}–10^{-5} M) did not increase (Ca^{2+}–

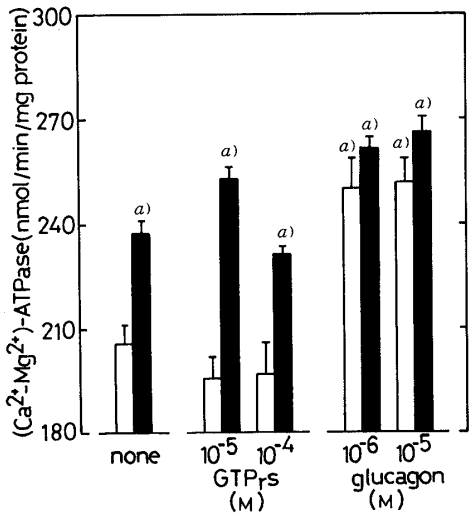

Fig. 6. Effect of GTP_rS and Glucagon on Regucalcin-Increased (Ca^{2+}–Mg^{2+})-ATPase Activity in the Plasma Membranes of Rat Liver

Enzyme activity was measured in the reaction mixture containing 1.0 μM regucalcin and GTP_rS (10^{-5} and 10^{-4} M) or glucagon (10^{-6} and 10^{-5} M). Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the control (none) value. □, control (absence of regucalcin); ■, presence of regucalcin.

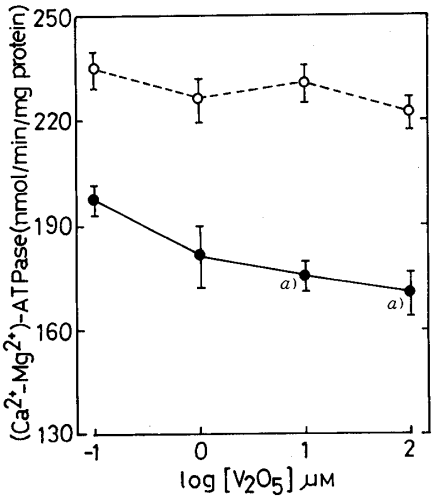

Fig. 7. Effect of Vanadium on Regucalcin-Increased (Ca^{2+}–Mg^{2+})-ATPase Activity in the Plasma Membranes of Rat Liver

The enzyme activity was measured in the reaction mixture containing 1.0 μM regucalcin and/or vanadium (0.1—100 μM). Addition of 0.1 or 1.0 μM vanadium did not significantly decrease the enzyme activity. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the control (none) value. ---○---, in the presence of regucalcin; —●—, absence of regucalcin.

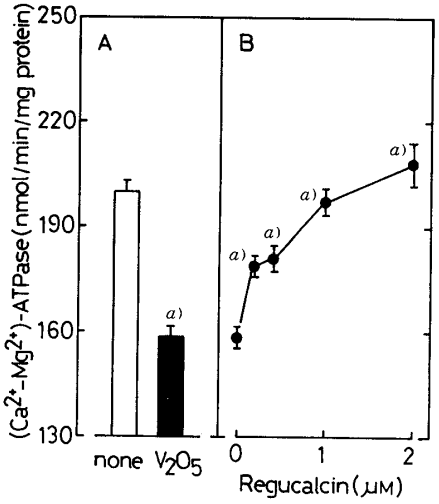

Fig. 8. Effect of Increasing Concentrations of Regucalcin on Vanadium-Decreased (Ca^{2+}–Mg^{2+})-ATPase Activity in the Plasma Membranes of Rat Liver

Figure A shows the effect of 100 μM vanadium on (Ca^{2+}–Mg^{2+})-ATPase activity. Figure B shows the effect of increasing concentrations of regucalcin on (Ca^{2+}–Mg^{2+})-ATPase activity in the presence of 100 μM vanadium. Each value represents the mean ± S.E.M. of 5 experiments. *a*) $p<0.01$, as compared with the control (none) value or with the vanadium alone.

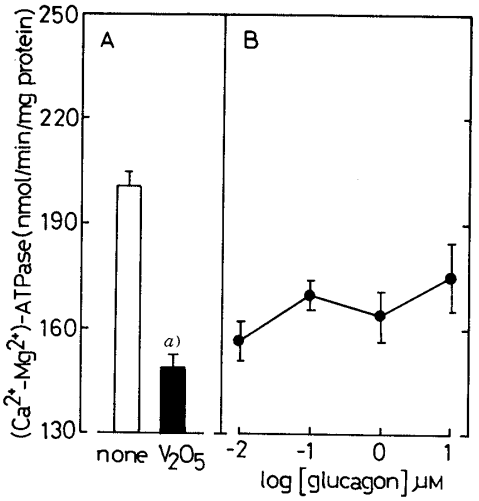

Fig. 9. Effect of Glucagon on Vanadium-Decreased (Ca^{2+}–Mg^{2+})-ATPase Activity in the Plasma Membranes of Rat Liver

Figure A shows the effect of 100 μM vanadium on (Ca^{2+}–Mg^{2+})-ATPase activity. Figure B shows the effect of increasing concentrations of glucagon on (Ca^{2+}–Mg^{2+})-ATPase activity in the presence of 100 μM vanadium. Each value represents the mean ± S.E.M. of 5 experiments. *a*) $p<0.01$, as compared with the control (none) value.

Mg^{2+})-ATPase activity. Also, the effect of regucalcin on the enzyme was not regulated by norepinephrine (data not shown).

Effect of Vanadium on Regucalcin-Increased (Ca^{2+}–Mg^{2+})-ATPase Activity in Liver Plasma Membranes

The effect of vanadium on (Ca^{2+}–Mg^{2+})-ATPase activity in the plasma membranes of rat liver is shown in Fig. 7. The enzyme activity was significantly decreased by the presence of vanadium (10 and 100 μM). The inhibitory effect of vanadium on the enzyme activity was not seen in the presence of 1.0 μM regucalcin. Vanadium (100 μM)-decreased (Ca^{2+}–Mg^{2+})-ATPase activity was significantly restored by the presence of 0.2 μM regucalcin (Fig. 8). With greater concentrations of regucalcin (1.0 and 2.0 μM), the recovery was complete.

The effect of glucagon on vanadium-decreased (Ca^{2+}–Mg^{2+})-ATPase activity in the plasma membranes of rat liver is shown in Fig. 9. Glucagon (0.01—10 μM) did not have a restorative effect on vanadium (100 μM)-decreased (Ca^{2+}–Mg^{2+})-ATPase activity, although the hormone (1 and 10 μM) caused a significant increase of the enzyme activity in the plasma membranes not treated with vanadium (Fig. 6).

Discussion

Regucalcin, a calcium-binding protein isolated from rat liver cytosol,[6,13] could bind to the plasma membrane, cytosol, mitochondrial, microsomal and nuclear fractions of rat liver cells. In particular, a large amount of ^{125}I-regucalcin bound to the plasma membranes, indicating that regucalcin, which existed in the cytosol of liver cells, may be bound to the plasma membranes. The binding of regucalcin to the plasma membranes was not dependent on the presence of Ca^{2+}. This indicates that regucalcin, which did not bind Ca^{2+}, could directly bind to the plasma membranes. At present, the binding site of regucalcin on the plasma

membranes is unknown. Regucalcin may be able to bind to the membrane proteins, because regucalcin can affect (Ca^{2+}–Mg^{2+})-ATPase activity in rat liver plasma membranes. However, the activity of the plasma membrane enzyme 5′-nucleotidase was not altered by regucalcin itself.[13] Presumably, the binding of regucalcin to the plasma membrane proteins(s) may be specific. It is possible, however, that regucalcin binds to the lipid component at least in part.

Regucalcin significantly increased plasma membrane (Ca^{2+}–Mg^{2+})-ATPase activity. This protein has 6—7 high-affinity binding sites per molecule of protein.[7] The Ca^{2+} binding constant was found to be $4.19 \times 10^5\ M^{-1}$ by equilibrium dialysis; the value of K_d was 2.4 μM. The concentration of free Ca^{2+} in the enzyme reaction mixture corresponded to 1.76 μM. It seems that Ca^{2+} bound to regucalcin dissociates from the Ca^{2+}-binding protein in the reaction mixture. Accordingly, it is unlikely that regucalcin binds Ca^{2+} of increase (Ca^{2+}–Mg^{2+})-ATPase activity. Furthermore, regucalcin stimulated the enzymatic hydrolysis of ATP in the absence of Ca^{2+} (Fig. 5). This strongly suggests that regucalcin directly binds to (Ca^{2+}–Mg^{2+})-ATPase and activates the enzyme.

It has been reported that GTP-binding protein in plasma membrane may play a role in the action of Ca^{2+}-mobilizing hormones on liver plasma membranes.[27-29] GTP_rS did not significantly alter (Ca^{2+}–Mg^{2+})-ATPase activity in rat liver plasma membranes. Also, the effect of regucalcin on the enzyme activity was not regulated by GTP_rS. These results suggest that the effect of regucalcin is not mediated through a GTP-binding protein located on liver plasma membranes. Norepinephrine, which binds to α_1- adrenergic receptors on the plasma membranes,[27] did not significantly alter (Ca^{2+}–Mg^{2+})-ATPase activity, and the hormone did not modify the effect of regucalcin on the enzyme activity (data not shown). α_1-Adrenergic receptors may be linked to GTP-binding protein in the hepatic membranes.[28,29] In addition, glucagon increased (Ca^{2+}–Mg^{2+})-ATPase activity in liver plasma membranes. However, glucagon did not enhance the increasing effect of regucalcin on the enzyme activity. These results further support the view that the effect of regucalcin on the membranous (Ca^{2+}–Mg^{2+})-ATPase is not coupled to GTP-binding protein.

It has been demonstrated that an ATP-dependent calcium-transport system exists in rat liver plasma membranes,[15] and the phosphorylated intermediate of the (Ca^{2+}–Mg^{2+})-ATPase is associated with this transport system. The calcium-dependent phosphorylation in the hepatic plasma membranes is inhibited by vanadium (100 μM).[15] In the present study, vanadium (10 and 100 μM) inhibited (Ca^{2+}–Mg^{2+})-ATPase activity. This may be based on an inhibition of the calcium-dependent phosphorylation caused by vanadium. The presence of regucalcin (2.0 μM) in the enzyme reaction mixture completely restored the decrease of (Ca^{2+}–Mg^{2+})-ATPase activity caused by vanadium (100 μM). This finding suggests that the regulatory mechanism by which regucalcin increases (Ca^{2+}–Mg^{2+})-ATPase activity is phosphorylation of the enzyme protein and/or direct activation of the enzyme, since regucalcin did not bind vanadium (data not shown). Meanwhile, glucagon did not weaken the inhibitory effect of vanadium on (Ca^{2+}–Mg^{2+})-ATPase. This indicates that the regulatory mechanism of regucalcin on the enzyme differs from the hormonal regulation.

It has been reported that calmodulin does not activate (Ca^{2+}–Mg^{2+})-ATPase in rat liver plasma membranes, and that there is an activator of the enzyme.[14] This activator is of a protein nature, although it has not yet been identified.[14] However, regucalcin, which can increase (Ca^{2+}–Mg^{2+})-ATPase activity, clearly differs from the activator as reported by Lotersztajn *et al.*[14]

Recently, we have reported that Ca^{2+} activates (dependently on, or independently of, calmodulin) the cytosolic fructose 1,6-bisphosphatase[9] and pyruvate kinase,[10] the particulate glycogen phosphorylase *a*[11] and the mitochondrial succinate dehydrogenase[12] in the liver of rats, and that the metal ion inhibits the plasma membrane 5′-nucleotidase.[13] The activation and inhibition of those enzymes caused by Ca^{2+} are completely reversed by the presence of

regucalcin.[9-13] The reversible effect of regucalcin is based on the characteristic that the protein can bind Ca^{2+}. Thus, regucalcin regulates the action of Ca^{2+} on liver cell functions. Furthermore, the novel finding that regucalcin can directly activate (Ca^{2+}–Mg^{2+})-ATPase, which functions as a Ca^{2+} pump[18] in rat liver plasma membranes, suggests that regucalcin may play a cell physiological role in the stimulation of the extrusion of increased cytosolic Ca^{2+} in liver cells.

In conclusion, it has been demonstrated that regucalcin binds to rat liver plasma membranes, and activates the (Ca^{2+}–Mg^{2+})-ATPase. The present findings further support the view that regucalcin plays an important role as a regulatory protein in liver cell functions related to Ca^{2+}

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