Hepatic Calcium-Binding Protein Regucalcin Decreases Ca$^{2+}$/Calmodulin-Dependent Protein Kinase Activity in Rat Liver Cytosol

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The effect of regucalcin, a calcium-binding protein isolated from rat liver cytosol, on cytosolic Ca$^{2+}$/calmodulin-dependent protein kinase activity was investigated. The increase in cytosolic Ca$^{2+}$/calmodulin-dependent protein kinase activity with passage of incubation time was clearly prevented by the presence of regucalcin (1.0 μM). An appreciable effect of regucalcin was seen at 0.5 μM. The cytosolic Ca$^{2+}$/calmodulin-dependent protein kinase activity was fairly increased by increasing concentrations of added Ca$^{2+}$ (0.25–1.0 mM). This increase was clearly blocked by the presence of regucalcin (1.0 μM). The inhibitory effect of regucalcin on the protein kinase activity was also seen with varying concentrations of calmodulin (2.5–15 μg). In the presence of regucalcin (1.0 μM), trifluoperazine (50 μM), an antagonist of calmodulin, significantly decreased the cytosolic Ca$^{2+}$/calmodulin-dependent protein kinase activity. These results suggest that regucalcin can regulate the Ca$^{2+}$/calmodulin effect in liver cytosol.

Keywords calcium; regucalcin; calmodulin; protein kinase; rat liver cytosol

It has been established that liver metabolism is regulated by the increase of Ca$^{2+}$ in the cytosol of liver cells due to hormonal stimulation. The Ca$^{2+}$ effect is modulated through calmodulin, a calcium-binding protein, in liver cells. Ca$^{2+}$ plays an important role in the regulation of liver cell function.

In recent years it has been reported that the calcium-binding protein (regucalcin), which differs from calmodulin, is distributed in the hepatic cytosol of rats. The molecular weight of regucalcin isolated from rat liver cytosol was estimated to be 28800, and the Ca$^{2+}$ binding constant was found to be $4.19 \times 10^4$ M$^{-1}$ by equilibrium dialysis. This novel protein has a reversible effect on the activation and inhibition of various enzymes by Ca$^{2+}$ in liver cells. Regucalcin may play a cell physiological role in the regulation of liver cell function related to Ca$^{2+}$.

On the other hand, Ca$^{2+}$/calmodulin-dependent protein kinase locates in rat liver cytosol. The present investigation, therefore, was undertaken to clarify whether regucalcin can regulate the cytosolic Ca$^{2+}$/calmodulin-dependent protein kinase activity. It was found that regucalcin decreases Ca$^{2+}$/calmodulin-dependent protein kinase activity in rat liver cytosol. The present finding may support the view that regucalcin plays a role as a regulatory protein for the Ca$^{2+}$/calmodulin-dependent effect in liver cells.

Materials and Methods

Chemicals Adenosine triphosphate (ATP), leupeptin hemisulfate, and calmodulin (from bovine brain) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calcium chloride, phenylmethylsulfonyl fluoride, trifluoperazine, and all other reagents were obtained from Wako Pure Chemical Co. (Osaka, Japan). [γ-32P]ATP (10 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). The reagents were dissolved in distilled water and then passed through ion-exchange resin to remove metal ions.

Isolation of Regucalcin Male Wistar rats, weighing 100–120 g, purchased from the Japan SLC Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca, and 1.1% P, and distilled water, freely. After one week on this diet, animals were killed by bleeding. The livers were perfused with Tris–HCl buffer (pH 7.4), containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4° C. The livers were removed, cut into small pieces, suspended 1:4 in Tris–HCl buffer (pH 7.4) and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 55000 g in a refrigerated centrifuge for 10 min and the supernatant was spun at 105000 g for 60 min. Regucalcin in the 105000 g supernatant (cytosol fraction) 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.

Assay of Ca$^{2+}$/Calmodulin-Dependent Protein Kinase Activity Rats were killed by cardiac puncture, and the liver was perfused with ice-cold 0.25 M sucrose, frozen immediately, cut into small pieces, suspended 1:4 in a 0.25 M sucrose solution containing 10 mM β-mercaptoethanol, 1 mM ethyleneglycol bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, and homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 5500 g in a refrigerated centrifuge for 10 min to remove mitochondria. The 5500 g supernatant was spun at 105000 g for 60 min, and the supernatant fraction (cytosol) was pooled to assay Ca$^{2+}$/calmodulin-dependent protein kinase activity.

Ca$^{2+}$/calmodulin-dependent protein kinase activity was measured at 30° C in an incubation volume of 50 μl as described by Connell et al. Phosphorylation of the substrate was performed in a reaction mixture containing liver cytosol (490–540 μg of protein), 50 mM HEPES, pH 7.4, 5 mM MgCl$_2$, 0.2 mM EGTA, 20 μM ATP, 0.5 μM [γ-32P]ATP and unless otherwise indicated, 0.5 mM CaCl$_2$, 5.0 μg of calmodulin and regucalcin (0.1–2.0 μM). The phosphorylation reaction was terminated by the addition of 2 ml of ice-cold 5% trichloroacetic acid containing 10 mM H$_3$PO$_4$. The radioactivity retained on GF/B glass fiber filters after filtration was determined by counting the dried filters in 2 ml of scintillation fluid. Ca$^{2+}$/calmodulin-dependent protein kinase activity was determined after subtracting the incorporation in the absence of Ca$^{2+}$ and calmodulin. The enzyme activity was expressed as the radioactivity (cpm) of [γ-32P]phosphate phosphorylated per min per mg of the cytosolic protein.

Protein concentration was determined by the method of Lowry et al. using bovine albumin as a standard.

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 alteration of Ca$^{2+}$/calmodulin-dependent protein kinase activity in rat liver cytosol with increasing incubation time is shown in Fig. 1. The enzyme reaction mixture contained both calmodulin (5.0 μg) and CaCl$_2$ (0.5 mM) added. The enzyme activity markedly increased with the passage of incubation time. This increase was clearly prevented by the presence of regucalcin (1.0 μM). The inhibitory effect of regucalcin was seen at 5.0 min of incubation. At 20 min of incubation, the inhibition by regucalcin was about 50% of control. Regucalcin was not...
phosphorylated by endogenous kinase and did not have kinase activity (data not shown).

The effect of increasing concentrations of regucalcin on Ca\(^{2+}\)/calmodulin-dependent protein kinase activity in rat liver cytosol is shown in Fig. 2. An appreciable effect of regucalcin on the enzyme activity was not seen at 0.1 \(\mu\)M. However, the enzyme activity was significantly decreased by the presence of 0.5 \(\mu\)M regucalcin. With the greater concentrations (1.0 and 2.0 \(\mu\)M), the effect was saturated.

When the concentration of Ca\(^{2+}\) added in the enzyme reaction mixture was varied in the range of 0.25 to 1.0 \(\text{mM}\) with 5.0 \(\mu\)g calmodulin, Ca\(^{2+}\)/calmodulin-dependent protein kinase activity in the liver cytosol increased dependent of Ca\(^{2+}\) concentration (Fig. 3). In the presence of 0.25 \(\text{mM}\) Ca\(^{2+}\) added into the enzyme reaction mixture containing 0.2 \(\text{mM}\) EGTA, regucalcin (1.0 \(\mu\)M) completely prevented an increase in the cytosolic Ca\(^{2+}\)/calmodulin-dependent protein kinase activity. The inhibitory effect of regucalcin (1.0 \(\mu\)M) was seen at the greater concentrations (0.5—1.0 \(\text{mM}\)) of added Ca\(^{2+}\).

In the presence of 0.5 \(\text{mM}\) Ca\(^{2+}\) added, protein kinase activity in liver cytosol was markedly increased by the presence of calmodulin (2.5—15.0 \(\mu\)g) (Fig. 4). This increase was saturated in the presence of 5.0 \(\mu\)g calmodulin. In the presence of 2.5 \(\mu\)g calmodulin, the inhibitory effect of regucalcin on protein kinase activity was complete. With the greater concentrations (5.0—15.0 \(\mu\)g) of calmodulin, the effect of regucalcin was weakened.

The presence of trifluoperazine, an antagonist of calmodulin, caused a significant decrease of Ca\(^{2+}\)/calmodulin-dependent protein kinase activity in liver cytosol (Fig. 5). This inhibitory effect was remarkable at 50 \(\mu\)g trifluoperazine. In the presence of trifluoperazine (10—50 \(\mu\)g), regucalcin (1.0 \(\mu\)M) further enhanced the trifluoperazine-induced decrease in the cytosolic Ca\(^{2+}\)/calmodulin-dependent protein kinase activity.
the regucalcin-induced decrease of Ca^{2+}/calmodulin-dependent protein kinase activity in liver cytosol was also examined. The enzyme reaction mixture contained a constant concentration of added Ca^{2+} (0.5 mM). At the addition of 2.5 μg calmodulin, the presence of regucalcin completely blocked the increase of Ca^{2+}/calmodulin-dependent kinase activity in liver cytosol. With the greater concentrations of calmodulin (5.0—15.0 μg), the effect of regucalcin was weakened, though the inhibitory effect was seen. From these results, it appears that the effect of regucalcin to decrease the cytosolic Ca^{2+}/calmodulin-dependent protein kinase activity results from the indirect effect rather than the direct action on the enzyme. Presumably, regucalcin largely binds Ca^{2+} and inhibits the activation of Ca^{2+}/calmodulin-dependent protein kinase by Ca^{2+}.

The presence of trifluoperazine, an antagonist of calmodulin, caused a remarkable decrease of Ca^{2+}/calmodulin-dependent protein kinase activity in hepatic cytosol. Regucalcin slightly enhanced the inhibitory effect of trifluoperazine on Ca^{2+}/calmodulin-dependent protein kinase. Trifluoperazine can bind to the hydrophobic sites of the enzyme. The inhibitory effect of regucalcin on Ca^{2+}/calmodulin-dependent protein kinase activity from that of an antagonist of calmodulin. The effect of regucalcin may be based on the binding of Ca^{2+} by the protein.

At present, the physiological significance of regucalcin in inhibiting Ca^{2+}/calmodulin-dependent protein kinase activity in liver cytosol is not clearly understood. Garrison et al. showed that vassopressin acts on liver to phosphorylate seven cytoplasmic proteins through a Ca^{2+}-dependent pathway. The present finding, that regucalcin has an inhibitory effect on Ca^{2+}/calmodulin-dependent protein kinase in liver cytosol, suggests the possibility that regucalcin plays a role in the regulation of the Ca^{2+}/calmodulin-dependent protein kinase signal transduction pathway.

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References