Regulatory effect of regucalcin on (Ca$^{2+}$-Mg$^{2+}$)-ATPase in rat liver plasma membranes: comparison with the activation by Mn$^{2+}$ and Co$^{2+}$

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Abstract

The effect of various metals and regucalcin, a calcium-binding protein isolated from rat liver cytosol, on (Ca$^{2+}$-Mg$^{2+}$)-ATPase activity in the plasma membranes of rat liver was investigated. Of various metals (Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Co$^{2+}$ and Al$^{3+}$; 100 μM as a final concentration), Mn$^{2+}$ and Co$^{2+}$ increased markedly (Ca$^{2+}$-Mg$^{2+}$)-ATPase activity, while other metals had no effect. When Ca$^{2+}$ was not added into enzyme reaction mixture, Mn$^{2+}$ and Co$^{2+}$ (25–100 μM) did not significantly increase the enzyme activity, indicating that heavy metals act on Ca$^{2+}$-stimulated phosphorylation of the enzyme. Meanwhile, regucalcin (0.25–1.0 μM) caused a remarkable elevation of (Ca$^{2+}$-Mg$^{2+}$)-ATPase activity. This increase was not inhibited by the presence of 100 μM vanadate, although the effects of Mn$^{2+}$ and Co$^{2+}$ (100 μM) were inhibited by vanadate. Also, the inhibition of the Mn$^{2+}$ and Co$^{2+}$ effects by vanadate was not seen in the presence of regucalcin. Moreover, regucalcin (0.5 μM) increased significantly the enzyme activity in the absence of Ca$^{2+}$. This effect of regucalcin was not altered by increasing concentrations of Ca$^{2+}$ added, indicating that the regucalcin effect does not depend on Ca$^{2+}$. The present results suggest that regucalcin activates directly (Ca$^{2+}$-Mg$^{2+}$)-ATPase in liver plasma membranes, and that the activation is not involved in the Ca$^{2+}$-dependent phosphorylation of the enzyme. (Mol Cell Biochem 124: 169–174, 1993)

Key words: regucalcin, calcium-binding protein, (Ca$^{2+}$-Mg$^{2+}$)-ATPase, plasma membrane, rat liver

Introduction

Calcium ion (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 many 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 [2, 3]. In recent years, it has been reported that a calcium-binding protein, regucalcin, which differs from calmodulin, is distributed in the hepatic cytosol of rats [5, 6]. This novel protein has a reversible
effect on the activation and inhibition of various enzymes by Ca\(^{2+}\) and/or calmodulin in liver cells [7-10]. This protein probably plays an important role in the regulation of liver cell functions related to Ca\(^{2+}\).

In liver, the regulation of Ca\(^{2+}\) extrusion is poorly understood. The high-affinity (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase is located on the plasma membranes of rat liver [11,12]. This enzyme acts as a Ca\(^{2+}\) pump to extrude the metal ion from the cytosol of liver cells [12]. Regucalcin can increase (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase activity in the plasma membranes of rat liver [13]. This observation suggests that regucalcin plays a role in the regulation of Ca\(^{2+}\) pump activity in liver plasma membranes. However, the regulatory mechanism of regucalcin on (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase activity has not been clarified fully. The present investigation was undertaken to clarify a possible mechanism of regucalcin in the activation of (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase in rat liver plasma membranes. It was found that regucalcin activates directly (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase independent on Ca\(^{2+}\)-stimulated phosphorylation of the enzyme on liver plasma membranes.

**Materials and methods**

**Animals**

Male Wistar rats, weighing 100-120 g, were used. They were obtained commercially (Japan SLC Inc., 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.

**Chemicals**

Adenosine 5'-triphosphate (ATP) and ethyleneglycol-bis-(aminoethyl ether)N,N'-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). CaCl\(_2\)·2H\(_2\)O and 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**

Rats 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\(^{\circ}\) C). The livers were removed, cut into small pieces, suspended 1:4 in Tris-HCl buffer (pH 7.4) and the homogenate was spun at 5500 × g in a refrigerated centrifuge for 10 min and the supernatant was spun at 105000 × g for 60 min. The resulting supernatant was heated at 60\(^{\circ}\) C for 10 min and recentrifuged at 38000 × g for 20 min. Regucalcin in the supernatant was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion exchange chromatography on diethylaminoethyl-cellulose, as reported previously [5]. Protein concentration was determined by the method of Lowry et al. [14] using albumin as a standard.

**Preparation of liver plasma membranes**

The livers were removed after perfusion with ice-cold 0.25 M sucrose solution and placed in ice-cold medium containing 0.25 M sucrose, 5 mM Hepes-KOH, and 1 mM EGTA, pH 7.4. The liver plasma membranes were prepared according to the procedure of Prpić et al. [15]. Livers were minced with scissors and homogenized by 10 passes with a loose-fitting Dounce homogenizer followed by 3 passes with a tight-fitting homogenizer, then diluted to give a 6% (w/v) homogenate. The homogenate was then centrifuged at 1,464 × g for 10 min, and the resulting pellet was resuspended in the isolation medium and diluted to give a 6% (w/v) suspension. A volume (10.4 ml) of this was mixed with 1.4 ml of Percoll (Pharmacia) in 15-ml Cortex tubes and centrifuged at 34,540 × g for 30 min. Two distinct layers close to the top of the tube were revealed. These were harvested and washed in 5 volumes of 0.25 M sucrose, 50 mM Tris-HCl, pH 8.0, and the resulting pellets were resuspended in the same medium. Assay of marker enzymes (5'-nucleotidase, succinate dehydrogenase, glucose-6-phosphates, and RNA polymerase) showed that there was less than 5% contamination by nuclei, mitochondria, or microsomes. The protein content of the membranes was measured according to Lowry et al. [14].

**Assay of (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase**

(Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase in the plasma membranes was measured under conditions described by Lotersztajn et al., except that phosphate release was determined as described elsewhere [16]. The standard assay for (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase activity contained in a final volume of