Reversible Effect of Calcium-Binding Protein on the Ca\(^{2+}\)-Induced Activation of Succinate Dehydrogenase in Rat Liver Mitochondria

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The effect of calcium-binding protein (CaBP) isolated from rat liver cytosol on the Ca\(^{2+}\)-induced increase in succinate dehydrogenase activity of rat liver mitochondria was investigated. The liver mitochondrial succinate dehydrogenase activity was significantly increased by addition of Ca\(^{2+}\) in the range of 1.0—50 \(\mu\)M. Of various other metals (25 \(\mu\)M) tested, addition of Hg\(^{2+}\), Zn\(^{2+}\) and Cd\(^{2+}\) caused a significant decrease in succinate dehydrogenase activity of the hepatic mitochondria, while Mg\(^{2+}\), Mn\(^{2+}\) and Sr\(^{2+}\) had no effect; the increase in the enzyme activity was specific for Ca\(^{2+}\). This Ca\(^{2+}\)-induced increase in the mitochondrial succinate dehydrogenase activity was completely blocked by the presence of ruthenium red (10 and 50 \(\mu\)M), an inhibitor of mitochondrial Ca\(^{2+}\) uptake, indicating that Ca\(^{2+}\) was transported into the mitochondria and activated the enzyme there. In the presence of more than 7.0 \(\mu\)M CaBP, the Ca\(^{2+}\) (10 \(\mu\)M)-induced increase in succinate dehydrogenase activity was completely reversed, but 1.4 \(\mu\)M CaBP had no effect on this increase. CaBP itself (15 \(\mu\)M) did not have an inhibitory effect on the basal activity of succinate dehydrogenase. The present results suggest that CaBP regulates the increase in succinate dehydrogenase activity by blocking Ca\(^{2+}\) transport into the hepatic mitochondria of rats.

Keywords—calcium-binding protein; calcium ion; zinc ion; cadmium ion; mercuric ion; succinate dehydrogenase; hepatic mitochondria; hepatic cytosol

Introduction

Calcium ion (Ca\(^{2+}\)) plays an important role in the regulation of cell function.\(^1,2\) 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.\(^3,4\) Calmodulin, a Ca\(^{2+}\)-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 (CaBP), 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.\(^8-10\)

In liver cells, the cytosolic Ca\(^{2+}\) is transported into the mitochondria and the metal ion stimulates the mitochondrial function.\(^11,12\) Therefore, the present investigation was undertaken to clarify the effect of CaBP on the Ca\(^{2+}\)-induced stimulation of the hepatic mitochondrial function. Succinate dehydrogenase is located on the inner membranes of hepatic mitochondria and has an important role in the electron transport system. Here, we report that Ca\(^{2+}\) activates succinate dehydrogenase in the hepatic mitochondria, and we show that this Ca\(^{2+}\) effect is reversed by CaBP.

Materials and Methods

Animals—Male Wistar rats, weighing 100—120 g were used. They were obtained commercially (Nippon Bio
Supply Center, Tokyo, 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—2-(6-Iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride, ruthenium red and metallic salts (chloride form) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were of reagent grade. All water used was glass-distilled.

Isolation of CaBP—CaBP 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 Hepatic Mitochondria—The liver was perfused with an ice-cold 0.25 M sucrose solution and immediately cut into small pieces, suspended 1:4 in 0.25 M sucrose solution and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 600 g in a refrigerated centrifuge for 10 min and the supernatant was spun at 5500 g for 20 min to obtain the mitochondrial fraction. 13) The 5500 g pellet was washed twice with ice-cold 0.25 M sucrose solution by centrifugation for 10 min at 5500 g. The mitochondrial preparation for enzyme analysis was resuspended in ice-cold distilled water.

Analytical Methods—Enzyme assays were carried out under optimal conditions. Succinate dehydrogenase activity was measured by incubation of the reaction mixture for 15 min at 37 °C in a final volume of 1.0 ml containing 50 mm potassium phosphate (pH 7.4), 0.1% 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride, 50 mm sodium succinate, 25 mm sucrose and the mitochondrial protein (12–18 μg). 14) In separate experiments, the reaction mixture contained 0.1–100 μM Ca2+ and/or 10–50 μM ruthenium red or 0.7–15 μM CaBP (final concentrations). The reaction was stopped by addition of trichloroacetic acid (10%, 1.0 ml), then the formazan was extracted with 4.0 ml of ethyl acetate and its absorbance was measured at 490 nm. Enzyme activity was expressed as absorbance at 490 nm per min per mg protein. The protein concentration was determined by the method of Lowry et al. 15)

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 effect of Ca2+ addition on succinate dehydrogenase activity in mitochondria prepared from rat liver is shown in Fig. 1. The mitochondrial succinate dehydrogenase activity was significantly increased by addition of Ca2+ in the range of 1.0–50 μM. The maximal increase in the enzyme activity was seen at 10 μM Ca2+. With further increase in the concentration of Ca2+, the increase in succinate dehydrogenase activity declined. At 100 μM Ca2+, the enzyme activity showed no increase over the control value.

The effect of various metals on succinate dehydrogenase activity in the hepatic mitochondria is shown in Fig. 2. The enzyme activity was significantly decreased by the presence of Hg2+, Zn2+ and Cd2+ at a concentration of 25 μM, while Mg2+, Mn2+ and Sr2+ (25 μM) had no effect on the enzyme activity.

The effect of ruthenium red, a potent inhibitor of Ca2+ transport into the mitochondria, 16) on the Ca2+-induced increase in the mitochondrial succinate dehydrogenase activity is shown in Fig. 3. The presence of 10 and 50 μM ruthenium red had no inhibitory effect on the basal activity of succinate dehydrogenase. The Ca2+-induced increase in the enzyme activity,

![Fig. 1. Effect of Increasing Concentrations of Ca2+ on the Activity of Succinate Dehydrogenase in the Hepatic Mitochondria of Rats](image)

Addition of 10–1 μM Ca2+ 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 Ca2+ addition.
however, was completely blocked by the presence of ruthenium red (10 and 50 μM).

The effect of CaBP isolated from rat liver cytosol on the Ca²⁺-induced increase in the mitochondrial succinate dehydrogenase activity is shown in Fig. 4. Addition of Ca²⁺ (10 μM) caused a significant increase of succinate dehydrogenase in the hepatic mitochondria (Fig. 4A). This increase was completely reversed by the presence of CaBP at the concentration of 7.0 μM (Fig. 4B). This effect of CaBP was saturated at 15.0 μM. In the presence of less than 1.4 μM CaBP, the reversible effect was not seen. The effect of CaBP (7.0 μM) was also observed at 25 μM Ca²⁺ (data not shown). CaBP itself (15 μM) did not have an inhibitory effect on the basal activity of succinate dehydrogenase; the basal activity was 4.21 ± 0.23 (A₄₉₀ x 10⁻¹/min/mg protein) and in the case of CaBP addition the enzyme activity was 4.32 ± 0.34 (A₄₉₀ x 10⁻¹/min/mg protein) in five experiments.
Discussion

Succinate dehydrogenase located on the inner membranes of hepatic mitochondria is physiologically important in electron transport and oxidative phosphorylation. This enzyme activity was significantly increased by addition of Ca$^{2+}$ in the range of 1.0–50 μM; the maximal increase was seen at 10 μM Ca$^{2+}$. Of the various divalent ions used, Ca$^{2+}$ uniquely increased succinate dehydrogenase activity in the hepatic mitochondria; the enzyme activity was markedly decreased by addition of Hg$^{2+}$, Zn$^{2+}$ and Cd$^{2+}$ (25 μM). These results indicate that the mitochondrial succinate dehydrogenase is regulated by Ca$^{2+}$ in the physiological concentration range, although the mechanism by which Ca$^{2+}$ activates the enzyme is unknown. The present finding, that Ca$^{2+}$ can activate the mitochondrial succinate dehydrogenase, suggests a role of Ca$^{2+}$ in the regulation of the mitochondrial function.

Ruthenium red is a potent inhibitor of the transport of Ca$^{2+}$ into the hepatic mitochondria. The presence of ruthenium red (10 and 50 μM) completely prevented the increase in the mitochondrial succinate dehydrogenase activity by addition of Ca$^{2+}$ (10 μM); ruthenium red had no effect on the basal activity of the enzyme. This phenomenon may be a result of inhibition of Ca$^{2+}$ transport into the hepatic mitochondria by ruthenium red, since Ca$^{2+}$ added to the enzyme reaction mixture is transported into the hepatic mitochondria in the presence of succinate as the substrate. In fact, the presence of 1 and 3 mM adenosine triphosphate (ATP) did not enhance the increase in the mitochondrial succinate dehydrogenase activity caused by Ca$^{2+}$ addition (data not shown), although 3 mM ATP stimulates the transport of Ca$^{2+}$ into the hepatic mitochondria of rats. Presumably, Ca$^{2+}$ is transported into the hepatic mitochondria and activates succinate dehydrogenase in the inner membranes of mitochondria.

CaBP isolated from hepatic cytosol of rats reverses the activation of liver cytosolic enzymes by Ca$^{2+}$ at a cell physiological level. If CaBP disturbs the transport of Ca$^{2+}$ into the hepatic mitochondria, the increase in the mitochondrial succinate dehydrogenase activity caused by Ca$^{2+}$ addition may be weakened by the presence of CaBP in the enzyme reaction mixture. In fact, the increase in succinate dehydrogenase activity caused by Ca$^{2+}$ (10 μM) was completely blocked by the presence of more than 7.0 μM CaBP. This effect of CaBP was not seen at a concentration of less than 1.4 μM. In the previous report, it was shown that the mitochondrial transport of Ca$^{2+}$ in the presence of 1.4 μM CaBP was appreciably increased by addition of 3 mM ATP. It is possible that CaBP at higher concentrations can prevent the transport of Ca$^{2+}$ into the mitochondria, because of binding of Ca$^{2+}$ by the protein. The molecular weight of CaBP was estimated to be 28800, and the Ca$^{2+}$ binding constant was found to be 4.19 × 10$^5$ M$^{-1}$ by equilibrium dialysis. This protein has 6–7 high affinity binding sites per molecule. CaBP may bind Ca$^{2+}$ in the extramitochondrial region and weaken the effect of the metal on succinate dehydrogenase by blocking its transport into the hepatic mitochondria.

Thus far, it has been demonstrated that CaBP has a reversible effect on the activation of various enzyme by Ca$^{2+}$ in the liver cells of rats. CaBP differs from calmodulin, which modulates many biochemical effects of Ca$^{2+}$ in cells. CaBP may play a cell physiological role by controlling the stimulation of liver cell functions by Ca$^{2+}$, especially in the cytosol. It is proposed that CaBP is an important regulatory protein for liver cytosolic Ca$^{2+}$. Further investigations are needed to clarify the role of CaBP in liver cells.

References