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Regulatory Effect of Calcium-Binding Protein Isolated from Rat Liver Cytosol on Activation of Fructose 1,6-Diphosphatase by Ca^{2+}-Calmodulin

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The role of a calcium-binding protein (CaBP) isolated from rat liver cytosol was investigated in relation to the activation of hepatic fructose 1,6-diphosphatase by Ca^{2+}-calmodulin. Fructose 1,6-diphosphatase activity in rat liver cytosol was markedly increased by addition of Ca^{2+} (1.0—5.0 μM) to the incubation mixture. This increase was completely inhibited in the presence of *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W-7 15 μM), an inhibitor of calmodulin. Added Ca^{2+} (5.0 μM)-increased cytosolic fructose 1,6-diphosphatase activity was markedly enhanced by the coexistence of calmodulin (2.5 μg/ml). Further, fructose 1,6-diphosphatase isolated from rabbit liver cytosol was activated by Ca^{2+}-calmodulin. This activation was completely inhibited by CaBP (20 μg/ml) isolated from rat liver cytosol, though CaBP in the absence of calmodulin had no effect on liver fructose 1,6-diphosphatase activity. The present data suggest that CaBP can modify the action of Ca^{2+}-calmodulin in liver cells. It is proposed that CaBP, which may regulate Ca^{2+} effects on liver function, should be named calregulin.

Keywords——calcium-binding protein; fructose 1,6-diphosphatase; Ca^{2+}-calmodulin; rat liver cytosol; W-7

Introduction

In recent years, it has been demonstrated that the liver participates in the regulation of calcium metabolism due to the hepatic bile system in rats.[1,2] On the basis of this finding, a calcium-binding protein (CaBP) has been found in the cytosol of rat liver.[3–5] The molecular weight of CaBP isolated from rat liver cytosol was estimated to be 28800, and the calcium binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis.[5] Thus, this protein differs from calmodulin, which modulates many biochemical effects of Ca^{2+} in cells.[6] The cell physiological role of this protein, however, is not fully resolved, although it may be involved in intracellular homeostasis of Ca^{2+}.[7]

The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations.[8] Fructose 1,6-diphosphatase in the liver cytosol participates in the rate-controlling process of hepatic gluconeogenesis, and activation of this enzyme promotes the conversion from fructose 1,6-diphosphate to glucose-6-phosphate.[9] More recently, it has been found that hormonal action on fructose 1,6-diphosphatase in the hepatic cytosol of rats is mediated through Ca^{2+}.[10] It has not been clarified so far whether this enzyme is activated by Ca^{2+}-calmodulin. The present report describes the effect of Ca^{2+}-calmodulin on liver fructose 1,6-diphosphatase and clarifies the effect of CaBP on the enzyme activated by Ca^{2+}-calmodulin. It was found that the action of Ca^{2+}-calmodulin effect is inhibited by CaBP isolated from rat liver cytosol.

Materials and Methods

Animals——Male Wistar rats, weighing 100—130 g, were used. They were obtained commercially (Nippon Bio

Supplemental 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.

Chemicals and Enzyme——W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) was purchased from Rikaken Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). Fructose diphosphatase (D-fructose-1,6-phosphate 1-phosphohydrolase; EC 3.1.3.11) isolated from rabbit liver and calmodulin isolated from bovine brain were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Preparation of Liver Cytosol——The liver was perfused with ice-cold 0.25 M sucrose solution and frozen immediately, then cut into small pieces, which were suspended 1:4 in an ice-cold 0.25 M sucrose solution and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at $105000 \times g$ in a refrigerated centrifuge for 60 min and the supernatant fluid was collected to obtain the cytosol. The cytosolic calcium content was 0.55 ± 0.09 (nmol/mg protein; mean of five animals). Then, the cytosol was incubated with 10 μM EGTA (ethylene glycol bis (2-aminoethylether) *N,N,N',N'*-tetraacetic acid, pH 7.0) for 5 min at 4 °C in order to remove the calcium. This cytosolic fraction was used for enzyme analyses with a final concentration of 1.0 μM EGTA.

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.[4]

Analytical Methods——Enzyme assay was carried out under optimal conditions. Fructose 1,6-diphosphatase activity was assayed by the measurement of inorganic phosphate liberation.[11] The enzyme assay was adapted as follows. The usual test system contained 0.1 mM fructose 1,6-diphosphate, 50 mM Tris-HCl (pH 7.5), 20 mM 2-mercaptoethanol, 10 mM $MgSO_4$, 1 μM EGTA (pH 7.5), and the cytosol solution (containing 1.8—2.4 mg of protein) or rabbit liver fructose 1,6-diphosphatase (24 μg/ml), in a final volume of 1.0 ml. In some experiments, the test system contained W-7 (15 μM), calcium chloride (1—100 μM Ca^{2+}), calmodulin (5.0 μg/ml) and/or CaBP (0—50 μg/ml) in the above assay system. Assays were conducted by incubation for 15 min at 30 °C, and the reaction was stopped by addition of 0.2 ml of 30% trichloroacetic acid, then the precipitate was removed by centrifugation. The inorganic phosphate in the supernatant was determined according to the method of Nakamura and Mori.[12] The enzyme activity was expressed as nmoles of inorganic phosphate released per min per mg protein. The protein concentration was determined by the method of Lowry *et al.*[13]

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

Results

Effect of Ca^{2+}-Calmodulin on Liver Fructose 1,6-Diphosphatase

The effect of Ca^{2+} addition on fructose 1,6-diphosphatase activity in the cytosol prepared from rat liver is shown in Fig. 1. The cytosolic fructose 1,6-diphosphatase activity was significantly increased by addition of Ca^{2+} in the range of 1.0—10 μM. The maximal effect was observed at 5.0 μM Ca^{2+}. With higher levels of Ca^{2+}, the enzyme activity decreased. This increasing effect of Ca^{2+} on the enzyme activity was also observed in the presence of EGTA at a final concentration of 1.0 μM. Thus, addition of Ca^{2+} in the range of 1.0—5.0 μM clearly increased fructose 1,6-diphosphatase activity in the hepatic cytosol of rats.

The effect of W-7, a calmodulin inhibitor,[14] on fructose 1,6-diphosphatase activity in the hepatic cytosol was next examined, and the result is shown in Fig. 2. W-7 (15 μM) had no ef-

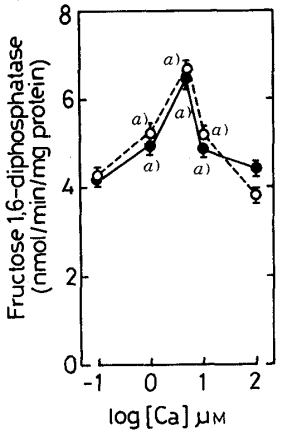

Fig. 1. Effect of Addition of Ca^{2+} on Fructose 1,6-Diphosphatase Activity in the Hepatic Cytosol of Rats

The cytosol was prepared from rat liver. The enzyme assay system containing the cytosolic enzyme was incubated with added Ca^{2+} in the range of 10^{-1}—10^{2} μM, and with or without 1.0 μM EGTA. Each point represents the mean of 5 animals. Vertical lines represent the S.E. ○---○, none; ●—●, 1.0 μM EGTA. *a)* $p<0.01$, as compared with the control value.

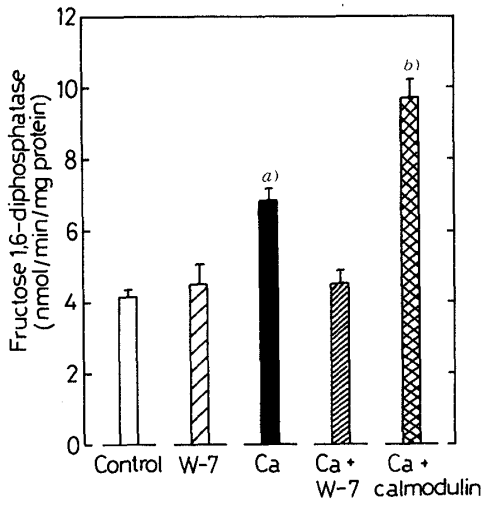

Fig. 2. Effect of Addition of Ca^{2+}, W-7, and Calmodulin on Fructose 1,6-Diphosphatase Activity in the Liver Cytosol of Rats

The cytosol was prepared from rat liver. The enzyme assay system containing the cytosol enzyme was incubated with 5.0 μM added Ca^{2+} and/or 15 μM W-7, and 5.0 μM added Ca^{2+} and calmodulin (5.0 μg/ml). Each point represents the mean of 5 animals. Vertical lines represent the S.E. *a)* $p<0.01$, as compared with the control value. *b)* $p<0.01$, as compared with Ca^{2+} addition value.

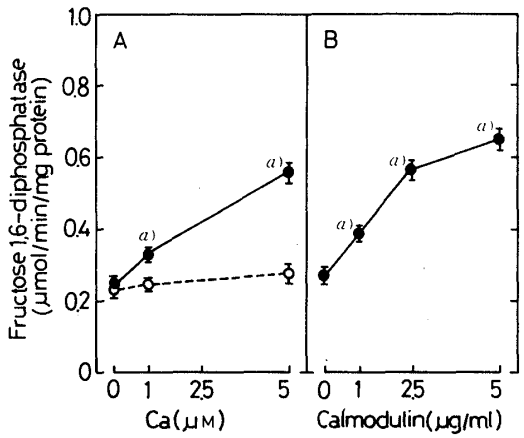

Fig. 3. Effects of Ca^{2+} and Calmodulin on Fructose 1,6-Diphosphatase Activity Isolated from Rabbit Liver

A: The enzyme assay system containing fructose 1,6-diphosphatase was incubated with added Ca^{2+} in the range of 1.0—5.0 μM, and with or without calmodulin (2.5 μg/ml). B: The enzyme assay system containing the enzyme was incubated with 5.0 μM added Ca^{2+} and calmodulin in the range of 1.0—5.0 μg/ml. Each point represents the mean of 5 experiments. Vertical lines represent the S.E. ○---○, none; ●—●, calmodulin. *a)* $p<0.01$, as compared with "none."

fect on fructose 1,6-diphosphatase activity in the cytosol without Ca^{2+} addition, while the drug produced a remarkable decrease in the fructose 1,6-diphosphatase activity increased by addition of 5.0 μM Ca^{2+}. These data suggest that the enzyme activity increase may involve calmodulin. This was confirmed by the result that addition of calmodulin (5.0 μg/ml) further enhanced fructose 1,6-diphosphatase activity in the presence of 5.0 μM Ca^{2+}.

Furthermore, the effects of Ca^{2+} and calmodulin on fructose 1,6-diphosphatase were examined by using the enzyme isolated from rabbit liver (Fig. 3). This enzyme activity was not significantly altered by addition of Ca^{2+} in the range of 1.0—5.0 μM Ca^{2+}. However, the activity was markedly increased by the further addition of calmodulin (2.5 μg/ml). In the presence of 5.0 μM Ca^{2+}, the enzyme activity was increased dose-dependently by calmodulin (1.0 and 2.5 μg/ml). Thus, purified liver fructose 1,6-diphosphatase is activated by Ca^{2+}-calmodulin.

Effect of CaBP on the Activation of Fructose 1,6-Diphosphatase by Ca^{2+}-Calmodulin

The effect of CaBP, isolated from the cytosol of rat liver,[4] on the activation of fructose 1,6-diphosphatase in the hepatic cytosol by addition of Ca^{2+} (5.0 μM) was examined, and the results are shown in Fig. 4. The increase in the enzyme activity by Ca^{2+} addition was reversed to the control level by CaBP, and the effect was saturated at the concentration of 20 μg/ml. This supports the view that the calcium added to the reaction mixture was bound to CaBP, because this protein has 6—7 high-affinity binding sites per molecule of protein.[5]

The effect of CaBP on the activation of fructose 1,6-diphosphatase by Ca^{2+}-calmodulin was examined by using the enzyme from rabbit liver. This enzyme activity was elevated about 3-fold by additions of both Ca^{2+} (5.0 μM) and calmodulin (5.0 μg/ml).

This increase was not observed in the presence of CaBP (20 μg/ml) in the reaction system, although the protein alone had no significant effect on fructose 1,6-diphosphatase (Fig. 5A).

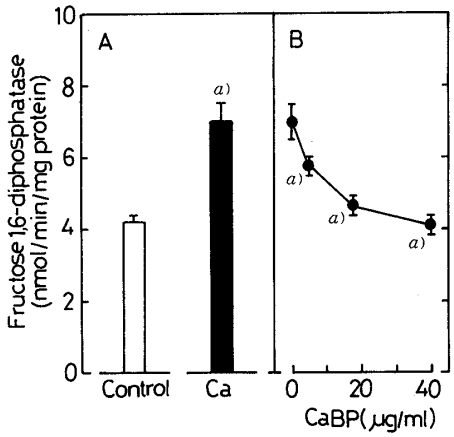

Fig. 4. Effect of Calcium-Binding Protein (CaBP) on Fructose 1,6-Diphosphatase Activity of Rat Liver Cytosol Increased by Addition of Ca^{2+}

The enzyme assay system containing the hepatic cytosol was incubated with 5.0 μM added Ca^{2+} (A), and with both 5.0 μM Ca^{2+} and CaBP in the range of 5.0—40 μg/ml (B). Each bar or point represents the mean of 5 animals. Vertical lines represent the S.E. *a*) $p<0.01$, as compared with the control value.

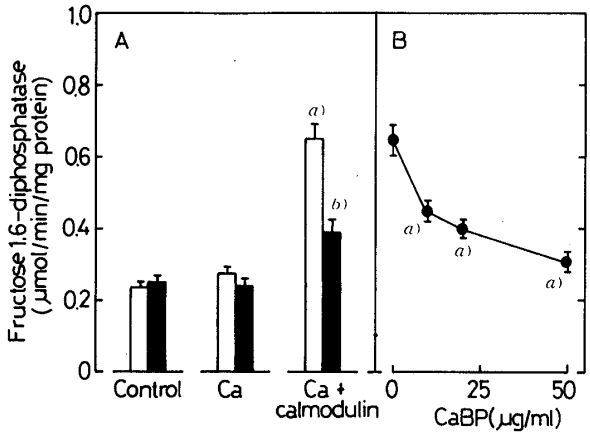

Fig. 5. Effect of Calcium-Binding Protein (CaBP) on the Activation of Fructose 1,6-Diphosphatase Isolated from Rabbit Liver by Ca^{2+}-Calmodulin

A: The enzyme assay system containing fructose 1,6-diphosphatase was incubated with 5.0 μM added Ca^{2+} and/or calmodulin (5.0 μg/ml) in the presence or absence of CaBP (20 μg/ml). B: The enzyme assay system containing fructose 1,6-diphosphatase was incubated with both 5.0 μM Ca^{2+} and calmodulin (5.0 μg/ml) in the presence of CaBP in the range of 10—50 μg/ml. Each bar or point represents the mean of 5 experiments. Vertical lines represent the S.E. □, none; ■, CaBP. *a*) $p<0.01$, as compared with the control value. *b*) $p<0.01$, as compared with CaBP "none" value.

The preventive effect of CaBP on the activation of fructose 1,6-diphosphatase by Ca^{2+}-calmodulin was enhanced by increasing concentrations of CaBP (Fig. 5B). This effect was saturated at a concentration of 50 μg/ml of CaBP. The concentration of CaBP required was about twice that calculated from the results in Fig. 4B. This difference may be because of the use of the purified enzyme.

Discussion

The calcium ion (Ca^{2+}) has many biological effects in liver cells,[8] and its effect is amplified through calmodulin.[6,15,16] Fructose 1,6-diphosphatase is the rate-limiting enzyme in gluconeogenesis in the hepatic cytosol, and the enzyme activity is regulated by hormones.[17] More recently, it was reported that this enzyme activity is increased by calcitonin, a calcium-regulating hormone, and that this hormonal action on the enzyme may be mediated through Ca^{2+}-calmodulin.[10] The present result, that liver fructose 1,6-diphosphatase is activated by Ca^{2+}-calmodulin, is a novel finding. Meanwhile, it is known that rat liver fructose 1,6-diphosphatase is inhibited by adenosine 5′-monophosphate and fructose 1,6-diphosphate.[11,18] This is an allosteric inhibition. It is unlikely that the effect of Ca^{2+}-calmodulin on this enzyme activation is related to the effect of those inhibitors, since the Ca^{2+}-calmodulin effect was completely inhibited by W-7, a calmodulin inhibitor.[14] The Ca^{2+}-calmodulin dependency of fructose 1,6-diphosphatase activity in liver cytosol suggests a cell physiological significance, since the enzyme is regulated by hormone.[10]

The activating effect of calmodulin on fructose 1,6-diphosphatase in liver cytosol was modified by CaBP isolated from rat liver cytosol.[4,5] This finding clearly indicates that CaBP can regulate the activation of calmodulin. The effect of CaBP reached a saturated level on the addition of increasing concentrations of CaBP to a reaction system containing both

calmodulin and Ca^{2+}. This indicates that CaBP does not inhibit fructose 1,6-diphosphatase activity. Although the mechanism by which CaBP modulates the calmodulin effect on the enzyme is unknown, CaBP may affect the binding of Ca^{2+} to calmodulin in hepatic cytosol. Which of the two proteins can bind Ca^{2+} may be decided by the relative concentrations of the two proteins and Ca^{2+} in hepatic cytosol, since the calcium-binding constant of CaBP is greater than that of calmodulin.[5,15]

Increased levels of Ca^{2+} in blood result in excretion of Ca^{2+} into the bile through the liver cells of rats.[2] Thus, a mechanism of intracellular transport of extracellular Ca^{2+} exists in liver cells.[2] CaBP in liver cytosol can bind Ca^{2+} that enters the cytosol, and the Ca^{2+} is transported into the mitochondria and the microsomes,[7] suggesting a possible role of CaBP in liver cells. An increase of Ca^{2+} in liver cytosol may affect enzymes in liver cells, and this might not be favorable from a cell physiological point of view. The present finding, that CaBP regulates the activation of liver fructose 1,6-diphosphatase by Ca^{2+}-calmodulin, suggests that CaBP present in hepatic cytosol can reduce the biological effect of cytosolic Ca^{2+} entering the cells. If the effect of Ca^{2+} is regulated by CaBP, this protein may be a kind of calcium-regulating protein in liver cells. It is therefore proposed to name CaBP calregulin. Further investigation is in progress to clarify the cell physiological role of this protein.

In conclusion, the present investigation clearly demonstrates that fructose 1,6-diphosphatase in liver cytosol is activated by Ca^{2+}-calmodulin, and that this activation is regulated by CaBP present in the cytosol. This suggests an important role of CaBP in regulating the effects of Ca^{2+} on cell function.

References

1) M. Yamaguchi, Y. Takei, and T. Yamamoto, *Endocrinology*, **96**, 1004 (1975).
2) M. Yamaguchi, "Calcium Endocrinology," ed. by Y. Yoshitoshi and T. Fujita, Chugai Igaku, Tokyo, 1980, pp. 169—183.
3) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **23**, 2418 (1975).
4) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **26**, 1915 (1978).
5) M. Yamaguchi and K. Sugii, *Chem. Pharm. Bull.*, **29**, 567 (1981).
6) R. H. Kretsinger, *CRC Crit. Rev. Biochem.*, **8**, 119 (1980).
7) M. Yamaguchi, *Chem. Pharm. Bull.*, **33**, 3390 (1985).
8) J. R. Williamson, R. H. Cooper, and J. B. Hoek, *Biochim. Biophys. Acta*, **639**, 243 (1981).
9) B. M. Pogell, A. Tanaka, and R. C. Siddons, *J. Biol. Chem.*, **243**, 1356 (1968).
10) M. Yamaguchi and K. Momose, *Horm. Metabol. Res.*, **16**, 650 (1984).
11) K. Taketa and B. M. Pogell, *J. Biol. Chem.*, **240**, 651 (1965).
12) M. Nakamura and K. Mori, *Nature* (London), **182**, 1441 (1958).
13) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) T. Tanaka and H. Hidaka, *J. Biol. Chem.*, **255**, 11078 (1980).
15) L. Z. Vaneldik, J. G. Zendegui, D. R. Marshak, and D. M. Watterson, *In. Rev. Cytol.*, **77**, 1 (1982).
16) W. Y. Cheung, *Biochem. Biophys. Res. Commun.*, **38**, 533 (1970).
17) S. Pontremoli, A. De Flora, F. Salamino, E. Melloni, and B. L. Horecker, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2969 (1975).
18) G. Weber, "Fructose 1,6-Diphosphatase and Its Role in Gluconeogenesis," ed. by R. W. McGilvery and B. M. Pogell, American Institute of Biological Science, Washington, D.C., 1964, p. 81.