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Effects of Ca^{2+} and Zn^{2+} on 5′-Nucleotidase Activity in Rat Liver Plasma Membranes: Hepatic Calcium-Binding Protein (Regucalcin) Reverses the Ca^{2+} Effect

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The effects of Ca^{2+} and Zn^{2+} on 5′-nucleotidase activity in rat liver plasma membranes were investigated. Addition of Ca^{2+} (1.0—100 μM) caused a significant decrease of the enzyme activity. The decrease of 5′-nucleotidase activity induced by Ca^{2+} (25 μM) was completely restored by the presence of a calcium-binding protein (CaBP 5.0 μM). Of the various metals tested, 5′-nucleotidase activity was significantly decreased by the presence of Zn^{2+} and Cd^{2+}, while Cu^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} did not inhibit the enzyme. In particular, the effect of Zn^{2+} was remarkable (decrease of about 60%). A significant decrease of the enzyme activity was also seen at 5.0 μM Zn^{2+}, but not at 1.0 μM Zn^{2+}. The inhibitory effect of 25 μM Zn^{2+} on 5′-nucleotidase was not appreciably blocked by the presence of CaBP (2.5 and 5.0 μM). The present data suggest that CaBP uniquely regulates the Ca^{2+} effect on 5′-nucleotidase activity in the hepatic plasma membranes. It is proposed that CaBP, which may regulate the Ca^{2+} effects on liver cell function, should be called regucalcin.

Keywords——calcium-binding protein; regucalcin; calcium; zinc; 5′-nucleotidase; rat liver plasma membrane

Introduction

It is well known that Ca^{2+} plays an important role in the regulation of many cell function.[1,2] The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations.[3,4] The Ca^{2+} effect is amplified through calmodulin in liver cells.[1,2] More recently, it has been found that a novel calcium-binding protein (CaBP) isolated from rat liver cytosol reverses the activating effect of Ca^{2+}-calmodulin on the liver cytosolic enzyme.[5] CaBP could also reverse the action of Ca^{2+} to activate many other enzymes in liver cells.[6–8] CaBP may play a cell physiological role different from that of calmodulin in the regulation of liver cell functions.

The present investigation was undertaken to investigate the reversing effect of CaBP on the actions of Ca^{2+} and other metals on 5′-nucleotidase (E.C. 3.1.3.5) in the hepatic plasma membranes of rat. The enzyme catalyzes the cleavage of phosphate from nucleoside 5′-monophosphates; it is an integral plasma membrane enzyme of most mammalian cells. It was found that the enzyme activity is decreased by Ca^{2+} and Zn^{2+}, and that the effect of Ca^{2+}, but not that of Zn^{2+}, is completely reversed by CaBP. The present results demonstrate that CaBP can reverse the inhibiting effect of Ca^{2+} on the liver enzyme, in addition to the activating actions of the metal. This further supports the view that CaBP can regulate Ca^{2+} action on liver cell functions. It is therefore proposed to call CaBP regucalcin.

Materials and Methods

Animals——Male Wistar rats, weighing 150—160 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.

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.[9]

Preparation of Liver Plasma Membranes——Rats were sacrificed under light ether anesthesia, and the liver was removed after perfusion with ice-cold 0.25 M sucrose solution. All subsequent procedures were carried out at 0—4 °C. The liver plasma membranes were prepared according to the procedure of Song *et al.*[10] 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.[11] A greater specific activity was found in fractions collected from the upper (B) interface, and contamination by mitochondria and microsomes was slight as estimated by means of assays for succinate dehydrogenase[12] and glucose-6-phosphatase,[13] respectively. Material from the upper (B) interface in each tube was 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 50 mM Tris–HCl buffer at pH 7.4. The protein concentration was determined by the method of Lowry *et al.*[14]

5′-Nucleotidase Assay——5′-Nucleotidase activity was assayed at pH 7.5 in presence of 10 mM $MgCl_2$ with 5′-adenosine monophosphate (5′-AMP) as the substrate.[11] The incubation mixture consisted of 50 mM Tris, 10 mM $MgCl_2$, 10 mM AMP and plasma membranes (50—60 μg as protein), with or without various metals and/or CaBP (1.0—5.0 μM) in a final volume of 1.0 ml. After a 10 min incubation at 37°C, the reaction was stopped by putting the mixture in a boiling water bath for 2 min, then the precipitate was removed by centrifugation. The inorganic phosphate in the supernatant was determined according to the method of Nakamura and Mori.[15]

Reagents——All reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and were dissolved in distilled water then passed through ion-exchange resin to remove metal ions.

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

The effect of Ca^{2+} addition on 5′-nucleotidase activity in the plasma membranes prepared from rat liver is shown in Fig. 1. The plasma membrane 5′-nucleotidase activity was significantly decreased by addition of Ca^{2+} in the range of 1.0—100 μM. As the concentration of Ca^{2+} was increased, the decrease of 5′-nucleotidase activity became greater. This decrease was also seen when the plasma membranes were washed with 1.0 mM EGTA (ethylene glycol bis(2-aminoethylether) *N*,*N*,*N*′,*N*′-tetraacetic acid, pH 7.0) (data not shown), suggesting that the plasma membrane 5′-nucleotidase is regulated by Ca^{2+}.

The effect of a CaBP isolated from the cytosol of rat liver on the decrease of 5′-nucleotidase activity in the hepatic plasma membranes caused by addition of Ca^{2+} (25 μM) was examined, and the results are shown in Fig. 2. The decrease of the enzyme activity caused by Ca^{2+} addition was reversed to the control level by the presence of CaBP (5.0 μM). CaBP itself did not have a significant effect on the enzyme activity of the control. 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.[16] The reversing effect of CaBP on the decrease in the hepatic plasma membrane 5′-nucleotidase activity caused by 25 μM Ca^{2+} addition was apparent in the presence of 2.5 μM CaBP, and the effect was saturated at the concentration of 5.0 μM.

The effect of various metals on 5′-nucleotidase activity in the hepatic plasma membranes

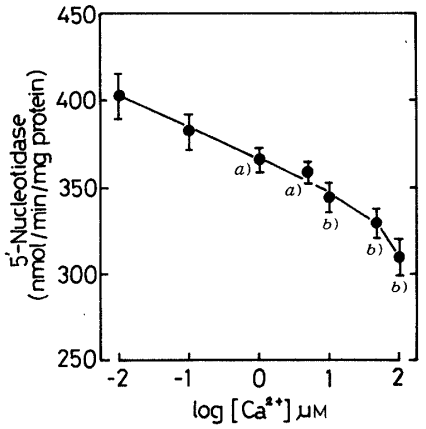

Fig. 1. Effect of Increasing Concentrations of Ca^{2+} on the Activity of 5'-Nucleotidase in the Hepatic Plasma Membranes of Rats

Addition of 10^{-2} or 10^{-1} μM Ca^{2+} did not alter the enzyme activity. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.05$ and *b)* $p<0.01$, as compared with the value without Ca^{2+}.

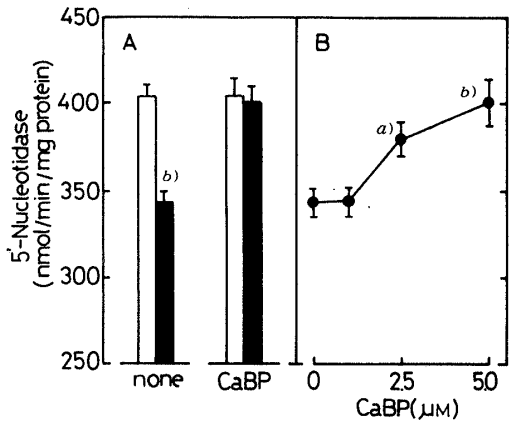

Fig. 2. Effect of CaBP on the Decrease of 5'-Nucleotidase Activity in Hepatic Plasma Membranes Caused by Ca^{2+} Addition

Figure A shows the effect of CaBP (5.0 μM) on the decrease of 5'-nucleotidase activity caused by addition of 25 μM Ca^{2+}. Figure B shows the effect of increasing concentrations of CaBP (1.0, 2.5 and 5.0 μM). Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.05$ and *b)* $p<0.01$, as compared with the control value. □, control; ■, 25 μM Ca^{2+} addition.

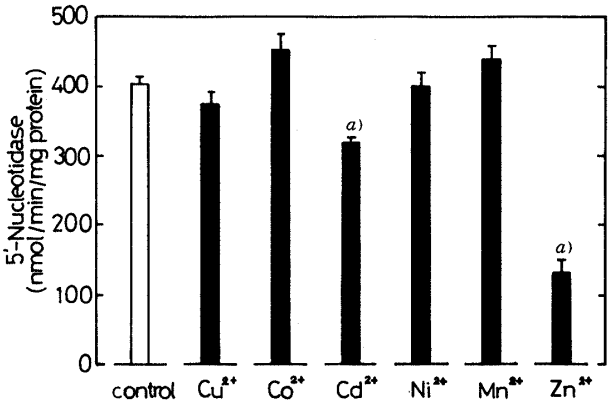

Fig. 3. Effect of Various Metals on 5'-Nucleotidase Activity in the Hepatic Plasma Membranes of Rats

The enzyme activity was measured in the reaction mixture containing 25 μM metal (final concentration). Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the control (none) value.

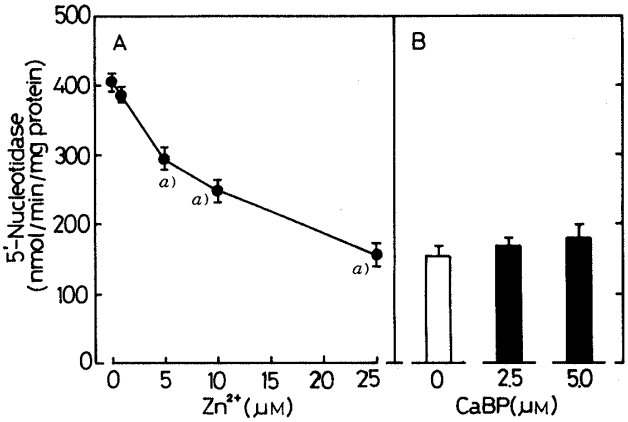

Fig. 4. Effect of CaBP on the Decrease of 5'-Nucleotidase Activity in the Hepatic Plasma Membranes Caused by Zn^{2+} Addition

Figure A shows the effect of increasing concentrations of Zn^{2+} on 5'-nucleotidase activity. Figure B shows the effect of CaBP (2.5 and 5.0 μM) on the decrease of 5'-nucleotidase activity caused by addition of 25 μM Zn^{2+}. Each value represents the mean ± S.E.M. of 5 experiments.

a) $p<0.01$, as compared with the control (none) value.

□, control; ■, presence of CaBP.

is shown in Fig. 3. The enzyme activity was significantly decreased by addition of 25 μM Cd^{2+} or Zn^{2+}, while Cu^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} at a concentration of 25 μM had no effect. The decrease of 5'-nucleotidase activity caused by Zn^{2+} addition was remarkable as compared with that of Cd^{2+}.

The effect of increasing concentrations of Zn^{2+} on 5'-nucleotidase activity in the hepatic plasma membranes is shown in Fig. 4A. The enzyme activity was significantly decreased by

addition of 5.0 μM Zn^{2+}. As the concentration of Zn^{2+} was increased, the decrease of 5′-nucleotidase activity became greater, and the effect seemed to be saturated at concentrations of over 25 μM Zn^{2+}. The effect of CaBP on the decrease of 5′-nucleotidase activity caused by addition of Zn^{2+} (25 μM) was examined (Fig. 4B). The decrease in the enzyme activity caused by Zn^{2+} addition was not significantly reversed by the presence of CaBP (2.5 and 5.0 μM). Thus, the reversing effect of CaBP on the decrease of 5′-nucleotidase activity was unique to Ca^{2+}.

Discussion

Recently, it has been reported that Ca^{2+} activates dependently on, or independently of, calmodulin the cytosolic fructose 1,6-bisphosphatase[5] and pyruvate kinase,[6] the particulate glycogen phosphorylase *a*[7] and the mitochondrial succinate dehydrogenase[8] in the liver of rats. The activation of those enzymes caused by Ca^{2+} is completely reversed by the presence of CaBP isolated from rat liver cytosol.[5-9] From these results, it is assumed that the liver cytosolic enzyme is more sensitive than the liver particulate enzyme to the action of CaBP to reverse the activation of enzymes by Ca^{2+} and/or calmodulin[5-8]; CaBP at 0.7 μM can completely reverse the activation of fructose 1,6-bisphosphatase by Ca^{2+}-calmodulin.[5] Furthermore, the present investigation demonstrates clearly that 5.0 μM CaBP can completely reverse the decrease of the hepatic plasma membrane 5′-nucleotidase activity caused by Ca^{2+}. This further supports the view that CaBP can regulate Ca^{2+} action on liver cell functions, and that the reversible effect of CaBP is reversed more sensitively in the case of the cytosolic enzyme than the particulate enzyme.

5′-Nucleotidase catalyzes the cleavage of phosphate from nucleoside 5′-monophosphates; it is an integral plasma membrane enzyme of most mammalian cells. The activity of 5′-nucleotidase in the hepatic plasma membranes of rats was significantly decreased by addition of Ca^{2+} in the range of 1.0—100 μM. This decrease may possibly be caused by the binding of Ca^{2+} to the plasma membranes, since there is a Ca^{2+}-binding site in the plasma membranes of rat liver[17,18]; the K_d value of the higher affinity binding sites of Ca^{2+} is in the range of 1.0—15 μM.[17,18] Ca^{2+} may be a regulator for 5′-nucleotidase in rat liver plasma membranes. The ability of Ca^{2+} (25 μM) to decrease 5′-nucleotidase activity in the hepatic plasma membranes was blocked by the presence of a CaBP isolated from rat liver cytosol. This effect of CaBP was appreciable at the concentration of 2.5 μM, and was complete at 5.0 μM. 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.[16] This protein has 6—7 high-affinity binding sites per molecule of protein.[16] At present, the mechanism of reversal of the Ca^{2+} effect on the enzyme is unknown. It is possible, however, that CaBP binds Ca^{2+} and thus weakens the effect of the metal.

Of various metal ions at a comparatively low concentration, Zn^{2+} caused a remarkable decrease of 5′-nucleotidase activity in the hepatic plasma membranes. This effect was appreciable at the concentration of 5.0 μM Zn^{2+}. This result is a novel finding, although it was reported that zinc activates human lymphoblast plasma membrane 5′-nucleotidase.[19] It is also suggested that zinc plays an important role in the maintenance of membrane structure and function.[20] The decrease of the hepatic plasma membrane 5′-nucleotidase activity caused by Zn^{2+} was not reversed by the presence of CaBP, whereas the protein blocked the effect of Ca^{2+} on the enzyme. This result indicates a specific regulatory action of CaBP on the Ca^{2+} effect.

More recently, it was reported that zinc binds to human brain calcium-binding proteins, calmodulin and S100b protein.[21] Calmodulin is characterized by two sets of Zn^{2+}-binding sites, with K_d ranging from 8×10^{-5} M to 3×10^{-4} M.[21] The S100b protein also exhibits two

sets of zinc-binding sites, with a much higher affinity ($K_d = 10^{-7} - 10^{-6}$ M).[21] A calcium-binding protein (called calregulin) which has 14 binding sites for Zn^{2+} with an apparent K_d value of 3.1×10^{-4} M is found in bovine liver cytosol,[22,23] although its cell-physiological function has not been clarified. This protein was characterized as M_r 63000 protein,[22] and clearly differs from our CaBP.[16,24] If CaBP could bind Zn^{2+}, the protein might exhibit a reversible effect on the Zn^{2+} action. It seems that CaBP does not bind Zn^{2+}.

The previous[5-8] and present investigations demonstrate that a CaBP isolated from rat liver cytosol can reverse the activation and inhibition of hepatic enzymes by Ca^{2+} and/or calmodulin. CaBP probably plays an important role in the regulation of Ca^{2+} action on liver cell functions. If the effect of Ca^{2+} is regulated by CaBP, this protein may be a kind of Ca^{2+} effect-regulating protein in liver cells. We therefore proposed to call CaBP regucalcin. Further investigation is in progress to clarify the role of this protein in liver cells.

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