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Reversible effect of calcium-binding protein regucalcin on the Ca^{2+}-induced inhibition of deoxyuridine 5′-triphosphatase activity in rat liver cytosol

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Abstract

The effect of regucalcin, a calcium-binding protein isolated from rat liver cytosol, on deoxyuridine 5′-triphosphatase (dUTPase) in the cytosol of rat liver was investigated. Addition of Ca^{2+} up to 5.0 μM to the enzyme reaction mixture caused a significant decrease of dUTPase activity, while Zn^{2+}, Cd^{2+}, Co^{2+}, Al^{3+}, Mn^{2+} and Ni^{2+} (10 μM) did not have an appreciable effect. The Ca^{2+}-induced decrease of dUTPase activity was reversed by the presence of regucalcin; the effect was complete at 1.0 μM of the protein. Regucalcin had no effect on the basal activity of the enzyme. Meanwhile, the reversible effect of regucalcin on the Ca^{2+} (10 μM)-induced decrease of dUTPase activity was not altered by the coexistence of Cd^{2+} or Zn^{2+} (10 μM). The present data suggest that liver cytosolic dUTPase is uniquely regulated by Ca^{2+} of various metals, and that the Ca^{2+} effect is reversed by regucalcin. (Mol Cell Biochem **110:** 25–29, 1992)

Key words: calcium, regucalcin, deoxyuridine 5′-triphosphatase, rat liver cytosol

Introduction

Recently, it has been demonstrated that liver metabolism is regulated by increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation [1, 2]. The Ca^{2+} effect is amplified through calmodulin, a calcium-binding protein, in liver cells [1, 2]. It has been also reported that a calcium-binding protein (regucalcin), which differs from calmodulin, is distributed in the hepatic cytosol of rats [3–5]. The molecular weight of regucalcin was estimated to be 28800, and the Ca^{2+} binding constant was found to be $4.19 \times 10^5 M^{-1}$ by equilibrium dialysis [4]. This novel protein has a reversible effect on the activation of various enzymes by Ca^{2+}/calmodulin in liver cells [6, 7]. Regucalcin may play a cell physiological role in the regulation of liver cell function related to Ca^{2+}.

On the other hand, the existence of deoxyuridine 5′-triphosphatase (EC 3.6.1.23; dUTPase), which catalyzes the conversion of dUTP to dUMP and PPi has been demonstrated in rat liver cytosol [8]. Although the function of this enzyme is still unclear, it has been

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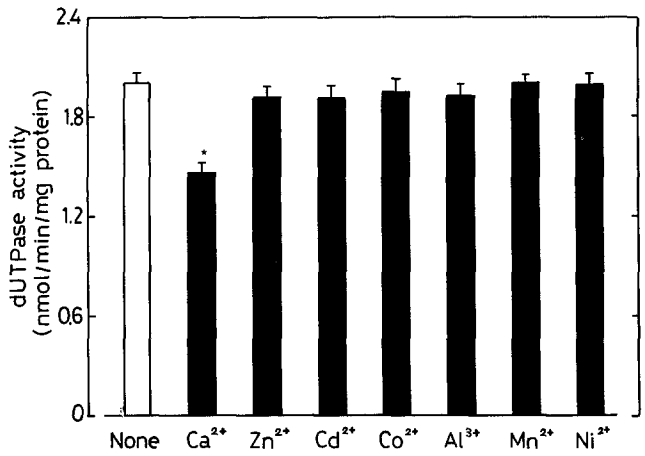

Fig. 1. Effect of various metal ions on dUTPase activity in the cytosol of rat liver. The enzyme activity was measured in the reaction mixture containing 10 μM metal as a final concentration. Each value represents the mean ± S.E.M. of 5 experiments. * $P < 0.01$, as compared with the control (none) value.

thought that it removes dUTP from the cells to prevent it from serving as a substrate for DNA polymerase [8], and also provides the substrate for thymidylate synthetase to synthesize TMP. Recently, it has been postulated that dUTPase serves to keep the concentration of dUTP low enough to avoid incoporation into DNA by DNA polymerase in liver cells [9].

Therefore, the present study was undertaken to investigate the regulatory effect of regucalcin on dUTPase in rat liver cytosol. It was found that, of various metal ions, the enzyme is uniquely inhibited by Ca^{2+}, and that regucalcin can reverse the Ca^{2+} effect. The present finding supports the view that regucalcin plays a regulatory role in the regulation of liver cell function by Ca^{2+}.

Materials and methods

Chemicals

dUTP, other nucleotides, and inorganic pyrophosphatase (from bahers yeast) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calcium chloride and all other reagents were obtained 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.

Animals

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.

Isolation of regucalcin

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 5500 g in a refrigerated centrifuge for 10 min and the supernatant was spun at 105000 g for 60 min. The supernatant was heated at 60° C for 10 min. The solution was then cooled and recentrifuged at 38000 × g for 20 min. The resulting supernatant fraction contained essentially regucalcin. 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 diethyl-aminoethyl (DEAE)-cellulose, as reported previously [3].

Assay of dUTPase activity

The livers were 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 1 mM ethyleneglycol bis(2-aminoethyl-ether)-N,N,N′,N′-tetra-acetic acid (EGTA), 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 dUTPase activity.

The assay of dUTPase activity was based on the release of PPi during incubation to inactivate inorganic pyrophosphatase (PPase) in the absence of Mg^{2+} [9]. The dUTPase reaction mixture (0.4 ml) contained 0.1 M Tris-HCl (pH 7.8), 0.180 mM dUTP, and liver cytosol (1.0–1.2 mg of protein). After incubation at 37° C for 20 min, the reaction was stopped by cooling in ice. Aliquots (0.2 ml) were transferred to another tube and 0.25 unit of PPase and 10 μl of 0.4 M $MgCl_2$ were added, followed by incubation for 5 min at 37° C to convert PPi to Pi. The Pi formed was determined by the method of Nakamura and Mori [10] after the termination of the reaction by addition of 5% trichloroacetic acid. The PPi formed in the main reaction mixture was determined and the difference calculated. The assay for

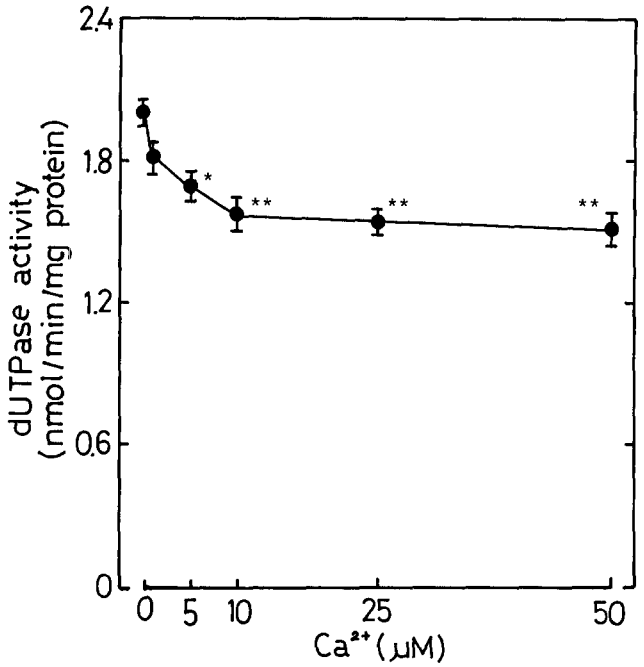

Fig. 2. Effect of increasing concentrations of Ca^{2+} on dUTPase activity in the cytosol of rat liver. The enzyme activity was measured in the reaction mixture containing 1.0, 5.0, 10, and 25 μM Ca^{2+} added as a final concentration. Addition of 1.0 μM Ca^{2+} did not affect the enzyme activity. Each value represents the mean ± S.E.M. of 5 experiments. * $P < 0.05$, and ** $P < 0.01$, as compared with the control (none) value.

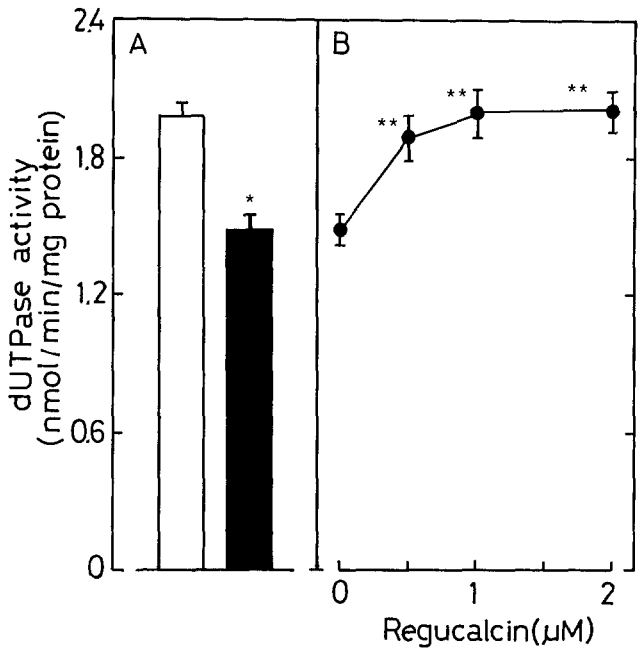

Fig. 3. Effect of regucalcin on the Ca^{2+}-induced decrease of dUTPase activity in the cytosol of rat liver. Figure A shows the effect of 10 μM Ca^{2+} addition on dUTPase activity. Figure B shows the effect of increasing concentrations of regucalcin (0.5, 1.0 and 2.0 μM) in the presence of 10 μM Ca^{2+}. Each value represents the mean ± S.E.M. of 5 experiments. * $P < 0.01$, as compared with the control (none) value. ** $P < 0.01$, as compared with the value of 10 μM Ca^{2+} addition without regucalcin. □, control; ■, 10 μM Ca^{2+} addition.

non-specific nucleotide triphosphate pyrophosphatase activity was carried out with UTP instead of dUTP as the substrate. Protein concentration was determined by the method of Lowry *et al.* [11]. The enzyme activity is expressed as nmol of PPi formed per min per mg of protein.

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 various metal ions on dUTPase activity in the cytosol of rat liver is shown in Fig. 1. The concentration of metal ions used in this experiment was 10 μM in the enzyme reaction mixture. Addition of Ca^{2+} caused a significant decrease in dUTPase activity. Zn^{2+}, Cd^{2+}, Co^{2+}, Al^{3+}, Mn^{2+} and Ni^{2+} did not have an appreciable effect on the enzyme activity. Thus, Ca^{2+} could uniquely regulate dUTPase in rat liver cytosol.

The effect of increasing concentrations of added Ca^{2+} on dUTPase activity in hepatic cytosol is shown in Fig. 2. Addition of 1.0 μM Ca^{2+} had no effect on the enzyme activity. Ca^{2+} up to 5.0 μM caused a significant decrease in the enzyme activity. At 10 μM Ca^{2+}, the decrease of the enzyme activity was maximum.

The effect of regucalcin, a calcium-binding protein, on dUTPase activity in hepatic cytosol is shown in Fig. 3. The presence of regucalcin (2.0 μM) in the enzyme reaction mixture did not have an appreciable effect on the basal activity of dUTPase (data not shown). The decrease of the enzyme activity caused by addition of Ca^{2+} (10 μM) was completely reversed by the presence of regucalcin (1.0 μM). At 0.5 μM regucalcin, the protein had an appreciable effect. The reversible effect of regucalcin was saturated at up to 1.0 μM of the protein.

In the presence of Ca^{2+} (5.0 and 10 μM), regucalcin had a reversible effect on the Ca^{2+}-induced decrease in dUTPase activity in hepatic cytosol (Fig. 4). At a higher

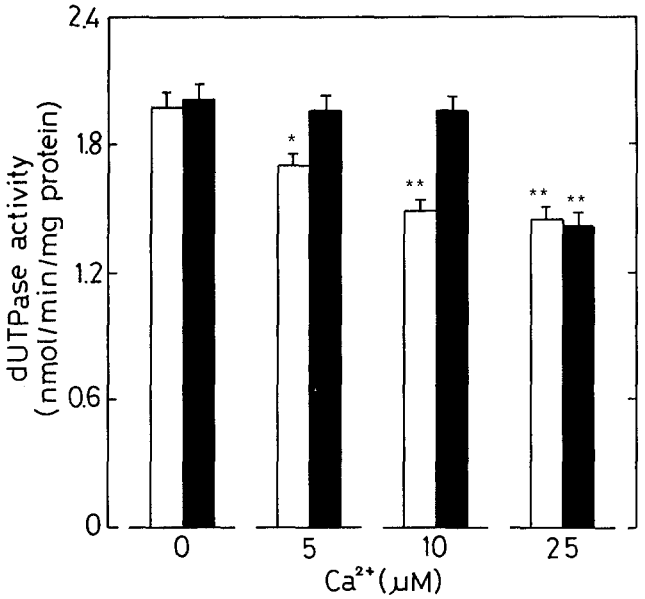

Fig. 4. Effect of increasing concentrations of Ca^{2+} on liver cytosolic dUTPase activity in the presence of regucalcin. The enzyme activity was measured in the reaction mixture containing 5.0, 10 and 25 μM Ca^{2+} (final concentration) in the presence or absence of 1.0 μM regucalcin. Each value represents the mean ± S.E.M. of 5 experiments. * $P < 0.05$, and ** $P < 0.01$, as compared with the control (none) value. □, control (none); ■, in the presence of 1.0 μM regucalcin.

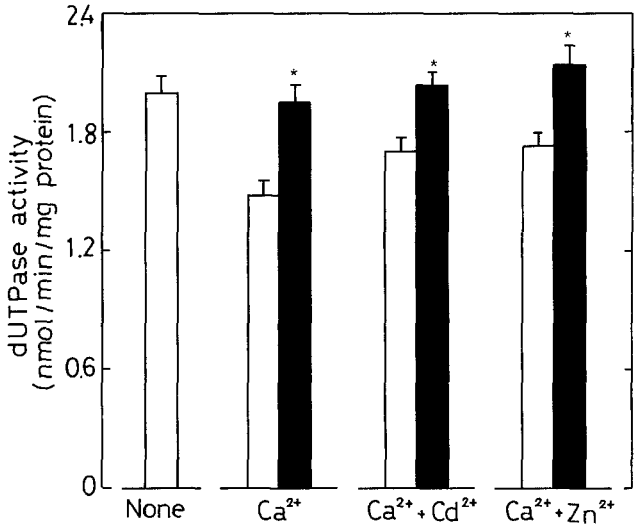

Fig. 5. Effect of regucalcin on liver cytosolic dUTPase activity in the presence of various metal ions. The enzyme activity was measured in the reaction mixture containing Ca^{2+}, Ca^{2+} plus Cd^{2+}, and Ca^{2+} plus Zn^{2+} (10 μM as final concentration) in the presence or absence of 1.0 μM regucalcin. Each value represents the mean ± S.E.M. of 5 experiments. * $P < 0.01$, as compared with the value obtained in the absence of regucalcin. □, without regucalcin; ■, with 1.0 μM regucalcin.

concentration of Ca^{2+} (25 μM), the effect of regucalcin (1.0 μM) was not seen.

An inhibitory effect of Ca^{2+} (10 μM) on dUTPase in hepatic cytosol was slightly weakened by the presence of Cd^{2+} or Zn^{2+} (10 μM) (Fig. 5). In the presence of Cd^{2+} or Zn^{2+} (10 μM), regucalcin (1.0 μM) had a reversible effect on the decrease in dUTPase activity by Ca^{2+}. Thus, the effect of regucalcin was not modified by the presence of Cd^{2+} or Zn^{2+} beside Ca^{2+}.

Discussion

Deoxyuridine 5′-triphosphatase (dUTPase) can catalyze the conversion of dUTP to dUMP and PPi. Although the function of this enzyme is fairly unclear, it has been thought that it removes dUTP from the cells to prevent it from serving as a substrate for DNA polymerase [8, 9], and also provides the substrate for thymidylate synthetase to synthesize TMP. Recently, it has been reported that dUTPase activity is increased after partial hepatoectomy at the same time of increase of DNA synthesis, suggesting that the enzyme activity is correlated with increased DNA synthesis [9]. Thus, dUTPase has a physiological role in liver cells.

In the present study, it was found that dUTPase activity in rat liver cytosol was significantly decreased by addition of Ca^{2+}, of various metal ions (10 μM). This decrease was seen at 5.0 μM Ca^{2+}, suggesting that the metal at physiological level has a regulatory role for dUTPase. Moreover, it was found that regucalcin, a calcium-binding protein isolated from rat liver cytosol, prevented the decrease of dUTPase activity in the hepatic cytosol by addition of Ca^{2+} (5.0 and 10 μM). The effect of regucalcin was complete at 1.0 μM. However, the regucalcin effect was not seen at a higher concentration of Ca^{2+} (25 M), when the protein exists at 1.0 μM in enzyme reaction mixture. Regucalcin did not have an appreciable effect on the basal activity of dUTPase in liver cytosol. The Ca^{2+} binding constant of regucalcin is found to be $4.19 \times 10^5\ M^{-1}$ by equilibrium dialysis, and there appear to be 6–7 high affinity binding sites for Ca^{2+} per molecule of protein [4]. Therefore, the effect of regucalcin to prevent the Ca^{2+}-induced decrease in dUTPase activity may be based on the binding of Ca^{2+}

due to regucalcin. Furthermore, the reversible effect of regucalcin on the Ca^{2+}-induced decrease in dUTOase activity in rat liver cytosol was clearly seen in the coexistence of Cd^{2+} or Zn^{2+} at the same concentration of Ca^{2+} (10 μM). This result suggests that regucalcin uniquely binds Ca^{2+}, and that the protein effect is not modified by the presence of Cd^{2+} and Zn^{2+}.

Presently, we do not know a physiological role of the reversible effect of regucalcin on the Ca^{2+}-induced decrease of dUTPase activity in rat liver cytosol. Since it has been postulated that dUTPase serves to keep the concentration of dUTP low enough to avoid incorporation into DNA by DNA polymerase [9], it is of interesting to note that regucalcin prevents a decrease in dUTPase activity due to Ca^{2+}, which may be increased in liver cell proliferation [12, 13]. The role of regucalcin in liver cell proliferation, however, remains to be elucidated, although there are growing evidences in the role of calmodulin [14, 15].

In conclusion, the present study demonstrates that Ca^{2+} uniquely decreases dUTPase activity in rat liver cytosol, and that regucalcin can reverse the effect of Ca^{2+}. The previous investigations showed that regucalcin can reverse activation of enzymes by Ca^{2+} and calmodulin [6, 7]. Presumably, regucalcin plays a regulatory role in liver cell function related to Ca^{2+}.

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References

1. Reinhart PH, Taylor WM, Bygrave FL: The role of calcium ions in the mechanism of action of α-adrenergic agonists in rat liver. Biochem J 223: 1–13, 1984
2. Williamson JR, Cooper RH, Hoek JB: Role of calcium in the hormonal regulation of liver metabolism. Biochim Biophys Acta 639: 243–295, 1981
3. Yamaguchi M, Yamamoto T: Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull 26: 1915–1918, 1978
4. Yamaguchi M, Sugii K: Properties of calcium-binding protein isolated from the soluble fraction of normal rat liver. Chem Pharm Bull 29: 567–570, 1981
5. Yamaguchi M, Mori S: Inhibitory effect of calcium-binding protein regucalcin on protein kinase C activity in rat liver cytosol. Biochem Med Metab Biol 43: 140–146, 1990
6. Mori S, Yamaguchi M: Hepatic calcium-binding protein regucalcin decreases Ca^{2+}/calmodulin-dependent protein kinase activity in rat liver cytosol. Chem Pharm Bull 38: 2216–2218, 1990
7. Yamaguchi M, Yoshida H: Regulatory effect of calcium-binding protein isolated from rat liver cytosol on activation of fructose 1,6-diphosphatase by Ca^{2+}-calmodulin. Chem Pharm Bull 33: 4489–4493, 1985
8. Bessmans MJ, Rehman IR, Adler J, Zimmerman SB, Simms FS, Kornberg A: Enzymatic synthesis of deoxyribonucleic acid. III. The incorporation of pyridine and purine analogues into deoxyribonucleic acid. Proc Natl Acad Sci USA 44: 633–640, 1958
9. Hokari S, Sakagishi Y, Tukada K: Enhanced activity of deoxyuridine 5′-triphosphatase in regenerating rat liver. Biochem Biophys Res Commun 108: 95–101, 1982
10. Nakamura M, Mori K: Colorimetric determination of inorganic phosphorus in the presence of glucose-1-phosphate and adenosin triphosphate. Nature 182: 1441–1442, 1958
11. Lowry OH, Rosebrough NJ, Farr AL, Randall FL: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–273, 1951
12. Boynton AL, Whitfield JF, Kleine LP: Ca^{2+}/phospholipid-dependent protein kinase activity correlates to the ability of transformed liver cells to proliferate in Ca^{2+}-deficient medium. Biochem Biophys Res Commun 115: 383–390, 1983
13. Cruise JL, Houck KA, Michalopoulos GK: Induction of DNA synthesis in cultured rat hepatocytes through stimulation of α_1 adrenoreceptor by norepinephrine. Science 227: 749–751, 1985
14. Serratosa J, Pujol MJ, Bachs O, Carafoli E: Rearrangement of nuclear calmodulin during proliferative liver cell activation. Biochem Biophys Res Commun 150: 1162–1169, 1988
15. Subramanyam C, Honn SC, Reed WC, Reddy GPV: Nuclear localization of 68 kDa calmodulin-binding protein is associated with the onset of DNA replication. J Cell Physiol 144: 423–428, 1990