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Inhibition of Ca^{2+}/Calmodulin-Dependent Phosphatase Activity by Regucalcin in Rat Liver Cytosol: Involvement of Calmodulin Binding

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Abstract The regulatory effect of regucalcin on Ca^{2+}/calmodulin-dependent phosphatase activity and the binding of regucalcin to calmodulin was investigated. Phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in rat liver cytosol was significantly increased by the addition of Ca^{2+} (100 μM) and calmodulin (0.30 μM). Thess increases were clearly inhibited by the addition of regucalcin (0.50–1.0 μM) into the enzyme reaction mixture. The cytosolic phosphoamino acid phosphatase activity was significantly elevated by the presence of anti-regucalcin monoclonal antibody (0.2 μg/ml), suggesting that endogenous regucalcin in the cytosol has an inhibitory effect on the enzyme activity. This elevation was prevented by the addition of regucalcin (0.50 μM). Purified calcineurin phosphatase activity was significantly increased by the addition of calmodulin (0.12 μM) in the presence of Ca^{2+} (1 and 10 μM). This increase was completely inhibited by the presence of regucalcin (0.12 μM). The inhibitory effect of regucalcin was reversed by the addition of calmodulin with the higher concentration (0.36 μM). Regucalcin has been demonstrated to bind on calmodulin-agarose beads by analysis with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The present study demonstrates that regucalcin inhibits Ca^{2+}/calmodulin-dependent protein phosphatase activity in rat liver cytosol, and that regucalcin can bind to calmodulin. J. Cell. Biochem. 71:140–148, 1998.

Key words: calmodulin; calcineurin; protein phosphatase; calcium-binding protein; regucalcin

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. The Ca^{2+} effect in cells is amplified by calmodulin, which is related to a signal transduction due to hormonal stimulation [Bygrave and Benedetti, 1993; Kraus-Friedman and Feng, 1996; Heizmann and Hunziker, 1991]. Meanwhile, regucalcin, a calcium-binding protein that is mainly expressed in liver [Shimokawa and Yamaguchi, 1993a,b; Yamaguchi et al., 1996], has been demonstrated to have an inhibitory effect on the activation of various enzymes by Ca^{2+} and calmodulin in liver cells [Yamaguchi and Tai, 1991; Yamaguchi and Mori, 1990; Yamaguchi, 1992]. Regucalcin may play a regulatory role in liver cell function related to Ca^{2+}. The regulatory mechanism of regucalcin on calmodulin effect in liver cytoplasm, however, has not been fully clarified.

Calcineurin, a calmodulin-binding protein, has been shown to possess a Ca^{2+}-dependent and calmodulin-stimulated phosphatase activity in brain [Pallen and Wang, 1983; Tallent and Cheung, 1986]. The enzyme also exists in the cytoplasm of liver cells [Tallent and Cheung, 1986]. Whether regucalcin has a regulatory effect on calcineurine activity in liver cells is not clear so far. Therefore, the present study was undertaken to determine the effect of regucalcin on Ca^{2+}/calmodulin-dependent phosphatase activity related to calcineurine. It was found that regucalcin can inhibit Ca^{2+}/calmodulin-dependent phosphatase activity in rat liver cytoplasm, and that it binds to calmodulin.

MATERIALS AND METHODS

Chemicals

Calmodulin [52,000 units (U)/mg protein from bovine brain], calcineurin (from bovine brain),

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calmodulin-agarose beads (1.5 mg calmodulin per ml of packed gel) insolubilized on 4% beaded agarose, *o*-phospho-L-tyrosine, *o*-phospho-L-serine, and *o*-phospho-L-threonine were obtained from the Sigma Chemical Co. (St. Louis, MO). NHS-LC-Biotin was obtained from Pierce (Rockford, IL). Streptavidin-peroxidase conjugate was obtained from Tago, Inc. (Burlingame, CA). Calcium chloride and all other chemicals were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All reagents used were dissolved in distilled water then passed through an ion-exchange resin to remove metal ions.

Animals

Male Wistar rats, weighing 100–120 g, were used. They were obtained commercially from the Japan SLC Inc. (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca, and 1.1% P at a room temperature of 25°C and were allowed distilled water freely.

Isolation of Regucalcin

Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1993a]. Regucalcin was isolated from rat liver cytosol. 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, and suspended 1:4 (weight:volume) in Tris-HCl buffer (pH 7.4); the homogenate was spun at 5,500*g* in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000*g* for 60 min. The resulting supernatant was heated at 60°C for 10 min and recentrifuged at 38,000*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 (DEAE)-cellulose, as reported previously [Yamaguchi and Yamamoto, 1978].

Determination of Regucalcin and Calmodulin by ELISA

Detection of regucalcin in the liver cytosol was performed using an enzyme-linked immunoadsorbent assay (ELISA) [Yamaguchi and Isogai, 1993]. This assay system was specific for regucalcin. 96-well assay plates (Nunc-Imuno) were coated with 50 µl of anti-regucalcin IgG diluted in 0.1 M carbonate buffer (pH 9.7) to a final concentration of 10 µg/ml. The plates were incubated with a standard amount of regucalcin (0.5–10 ng/ml, 50 µl) or liver cytosol (50 µl) for 18 h at 4°C. After wash, biotinylated anti-regucalcin IgG with NHS-LC-Biotin was added. Plates were then incubated for 2 h at 37°C and washed. Plates were incubated with streptavidin-peroxidase conjugate (1/40,000) for 2 h at 37°C, washed, and finally incubated with *o*-phenylenediamine (3 mg/ml, 100 µl) for 15 min at room temperature. The reaction was stopped with 100 µl 4 N H_2SO_4, and the plate was read at 450 nm. Regucalcin concentration was calculated from the standard curve for the determination of regucalcin. Regucalcin concentration was expressed as µg per mg of liver cytosolic protein.

The concentration of calmodulin in the liver cytosol was determined using an ELISA KIT (Takara Syuzo, Co. Ltd., Kyoto, Japan). Calmodulin concentration was expressed as micrograms per milligram of liver cytosolic protein.

Anti-Regucalcin Antibody

Anti-regucalcin antibody was a monoclonal antibody raised against regucalcin prepared using standard methods. Mice (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg per animal of antigen (rat liver regucalcin) emulsified with Freund's complete adjuvant, and 19 days later antigen (0.25 mg/animal) was intraperitoneally injected with Freund's incomplete adjuvant. Animals were killed by bleeding 3 days after the last injection. Spleen cells were prepared from immunized mouse and fused into myeloma cells. Anti-regucalcin monoclonal antibody–producing cells (hybridoma cells) were obtained by screening. The IgG from hybridoma cells was isolated through protein A-agarose column (Sigma, St. Louis, MO).

Assay of Phosphatase Activity

Rats were killed by cardiac puncture, and the livers perfused with ice-cold 0.25 M sucrose solution were removed, frozen immediately, cut into small pieces, suspended 1:4 (weight:volume) in 0.25 M sucrose solution, and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 5,500*g* in a refrigerated centrifuge for 10 min to

remove mitochondria. The 5,500*g* supernatant was spun at 105,000*g* for 60 min, and the supernatant fraction (cytosol) was pooled to assay phosphatase activity.

Phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine was assayed at 30°C in 1.0 ml of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM dithiothreitol, 9 mM phosphoaminoacid, $CaCl_2$ (10^{-6}–10^{-4} M), calcineurin (0.5 µg/ml), or cytosolic enzyme (1.5 mg/ml), and with or without calmodulin (2.0 and 5.0 µg/ml) as reported elsewhere [Fruman et al., 1992]. Regucalcin (0.1–1.0 µM) was contained in the above reaction mixture. The enzyme reaction was terminated after 15 min by the addition of 1.0 ml of cold 10% trichloroacetic acid and centrifuged to precipitate protein. Released inorganic phosphate in the supernatant was quantified by the method of Nakamura and Mori [1958]. Results were expressed as nmoles of inorganic phosphate liberated per minute per milligram of cytosolic protein.

Protein concentration was determined by the method of Lowry et al. [1951].

Calmodulin-Agarose Binding Assay

Calmodulin was insolubilized on 4% beaded agarose (containing 1.5 mg calmodulin per milliliter of packed gel). Calmodulin-agarose beads were preactivated by washing three times in buffer (10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM $CaCl_2$). The beads were aliquoted into individual microcentrifuge tubes and spun, and all of the supernatant was removed. The reaction mixture (1.0 ml) containing packed beads (60 µl; 90 µg calmodulin) in the above buffer was incubated in the presence of regucalcin (10 µg/ml) and/or calcineurin (2.5 µg/ml) for 1 h at 4°C [Loh et al., 1996]. After centrifugation, the beads were then washed three times in wash buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, and protease inhibitors supplemented with 1 mM $CaCl_2$ or 5 mM EGTA) and once in 10 mM Hepes, pH 7.4, and then boiled in Laemmli reducing buffer. The samples were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis

SDS-PAGE was performed by the method of Laemmli [1970] with minor modifications. The electrophoresis was carried out using 12% polyacrylamide resolving gel and the discontinuous Tris-glycine buffer system. Twenty microliters of sample was dissolved in each same volume of SDS-loading buffer containing 4% SDS, 10% β-mercaptoethanol, and bromphenol blue (BPB) marker. The protein mixture was denatured by heating at 90°C for 2 min and applied to individual wells. Electrophoresis voltage was applied (8 V/cm for 3 h at room temperature) to the gel. After separation, proteins were simultaneously fixed with methanol-acetic acid and stained with Coomassie Brilliant Blue R250. Then, the gels were destained with methanol-acetic acid and stored in water containing 20% glycerol.

Statistical Analysis

Data were expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. A *P* value of less than .05 was considered to indicate statistically significant differences.

RESULTS

Effect of Regucalcin on Phosphatase Activity in Rat Liver Cytosol

Regucalcin is mainly expressed in the liver of rats [Shimokawa and Yamaguchi, 1993a; Yamaguchi and Isogai, 1993]. Calmodulin and calcineurin are present in liver tissues [Tallent and Cheung, 1986]. The effect of regucalcin on phosphotyrosine phosphatase activity in rat liver cytosol is shown in Figure 1. The enzyme reaction mixture contained either vehicle or exogenous calmodulin (5 µg/ml; 0.30 µM) in the presence of liver cytosol (1.5 mg protein/ml) without or with the addition of regucalcin (0.1, 0.25, 0.5, and 1.0 µM). As shown in Figure 1A, phosphotyrosine phosphatase activity was found in liver cytosol. The enzyme reaction mixture with liver cytosol (1.5 mg protein) contained 0.026 µM of endogenous calmodulin. The addition of calmodulin (5 µg/ml; 0.30 µM) caused a significant increase of phosphotyrosine phosphatase activity in liver cytosol. This increase was significantly inhibited by the addition of regucalcin (0.25–1.0 µM) (Fig. 1B). The effect of calmodulin addition was completely inhibited by 1.0 µM regucalcin. Meanwhile, the cytosolic phosphotyrosine phosphatase activity without calmodulin addition was not significantly altered by regucalcin addition (0.1–1.0 µM) (data

not shown). Liver cytosol contained 16 μg of regucalcin per 1 mg of the cytosolic protein; the enzyme reaction mixture contained 0.72 μM of endogenous regucalcin. Moreover, the addition of regucalcin (0.05 or 0.10 μM) in the enzyme reaction mixture caused a significant decrease in Ca^{2+}/calmodulin-dependent phosphatase activity toward phosphoserine or phosphothreonine used as the substrate (Fig. 2).

The effect of anti-regucalcin monoclonal antibody on phosphatase activity with various phosphoaminoacids as the substrate in rat liver cytosol is shown in Figure 3. Phosphatase activity for phosphoserine and phosphothreonine in addition to phosphotyrosine was found in liver cytosol without or with 100 μM Ca^{2+} addition. Phosphatase activities in the presence of Ca^{2+} (100 μM) were significantly increased by the presence of anti-regucalcin antibody (0.2 μg/ml). Meanwhile, the addition of mouse IgG protein (0.1 and 1.0 μg/ml) did not have a significant effect on phosphotyrosine phosphatase activity in rat liver cytosol (data not shown). The effect of anti-regucalcin antibody on phosphatase activity was completely blocked by the addition of regucalcin (0.50 μM; 16.7 μg/ml).

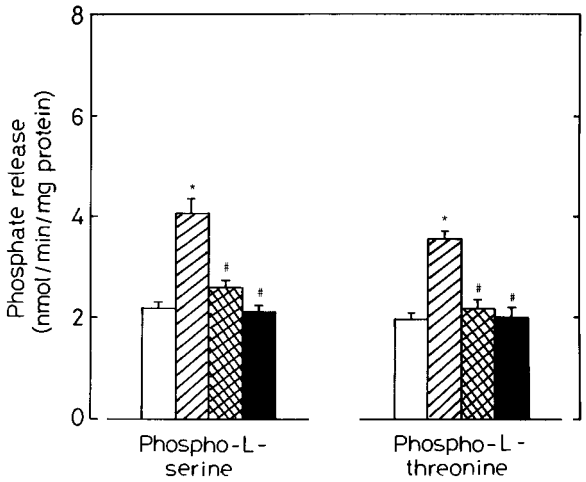

Fig. 2. Effect of regucalcin on Ca^{2+}/calmodulin-dependent phosphatase activity toward phosphoserine or phosphothreonine in rat liver cytosol. The enzyme reaction mixture contained either vehicle or calcium chloride (100 μM) plus calmodulin (5 μg/ml) in the absence or presence of regucalcin (0.5 and 1.0 μM). Each value represents the mean ± SEM of five experiments. *, $P < .01$, compared with the control value without Ca^{2+} and calmodulin addition. #, $P < .01$, compared with the value obtained from Ca^{2+} and calmodulin addition without regucalcin addition. Open bar: Control, hatched bar: Ca^{2+} and calmodulin addition, cross-hatched bar: Ca^{2+} and calmodulin addition with regucalcin (0.5 μM), solid bar: Ca^{2+} and calmodulin addition with regucalcin (1.0 μM).

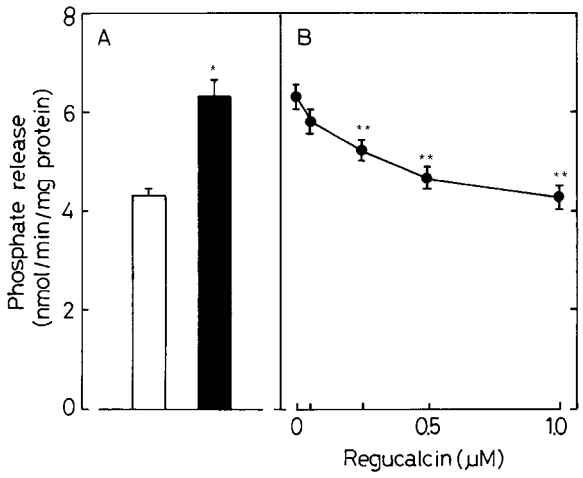

Fig. 1. Effect of regucalcin on Ca^{2+}/calmodulin-dependent phosphotyrosine phosphatase activity in rat liver cytosol. **A:** The enzyme reaction mixture contained either vehicle or calcium chloride (100 μM) plus calmodulin (5 μg/ml). **B:** The enzyme reaction mixture contained either vehicle or regucalcin (0.1, 0.25, 0.5, and 1.0 μM) in the presence of calcium chloride (100 μM) and calmodulin (5 μg/ml). Each value represents the mean ± SEM of five experiments. *, $P < .01$, compared with the control value without Ca^{2+} and calmodulin addition. **, $P < .01$, compared with the value without regucalcin addition. Open bar: Control, solid bar: Ca^{2+} and calmodulin addition.

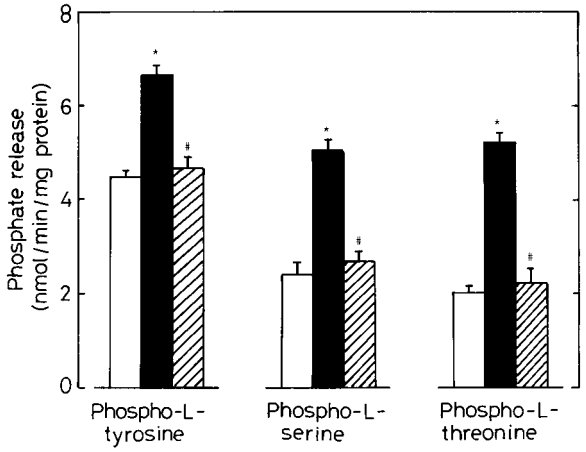

Fig. 3. Effect of anti-regucalcin antibody on phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in rat liver cytosol. The enzyme reaction mixture contained either vehicle, anti-regucalcin antibody (0.2 μg/ml), or anti-regucalcin antibody (0.2 μg/ml) plus regucalcin (0.50 μM) in the presence of each phosphoaminoacid with calcium chloride (100 μM) addition. Each value represents the mean ± SEM of five experiments. *, $P < .01$, compared with the control value without anti-regucalcin antibody addition. #, $P < .01$, compared with the value with anti-regucalcin antibody addition. Open bar: Control, solid bar: anti-regucalcin antibody addition, hatched bar: anti-regucalcin antibody plus regucalcin addition.

Thus, endogenous regucalcin had an inhibitory effect on phosphoaminoacid phosphatase activity in rat liver cytosol.

Effect of Regucalcin on Phosphatase Calcineurin Activity

Whether regucalcin has an effect on Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin activity was examined (Fig. 4). We used calcineurin isolated from bovine brain. The enzyme reaction mixture contained either vehicle, calmodulin (2.0 μg/ml; 0.12 μM), or calmodulin (2.0 μg/ml) plus regucalcin (0.12 μM) in the presence of calcineurin (0.5 μg/ml). Phosphotyrosine was used as the substrate for enzyme activity. Phosphatase activity in the presence of calmodulin was clearly increased by increasing Ca^{2+} concentrations (1 and 10 μM). This increase was completely inhibited by the presence of regucalcin. Moreover, phosphatase calcineurine activity toward phosphoserine or phosphothreonine used as the substrate was significantly increased by the addition of Ca^{2+} (10 μM) plus calmodulin (0.12 μM), as shown in Figure 5. These increases were prevented by the presence of regucalcin (0.12 μM).

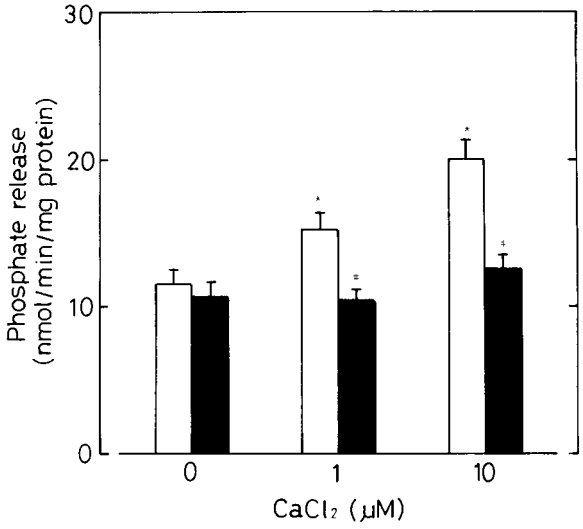

Fig. 4. Effect of regucalcin on Ca^{2+}/calmodulin-dependent phosphatase calcineurin activity toward phosphotyrosine. Calcineurin purified from rat brain was used in the enzyme assay. The reaction mixture contained either vehicle or regucalcin (0.12 μM) in the presence of both calcium chloride (1 or 10 μM) and calmodulin (2.0 μg/ml; 0.12 μM) with calcineurin (0.5 μg/ml). Each value represents the mean ± SEM of five experiments. *, $P < .01$, compared with the control value without calcium addition. #, $P < .01$, compared with the value without regucalcin addition. Open bar: without regucalcin, solid bar: with regucalcin.

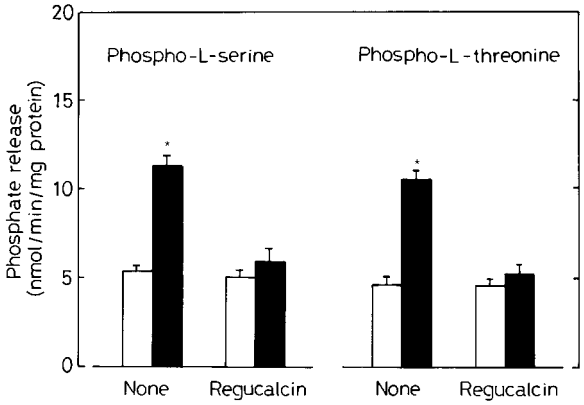

Fig. 5. Effect of regucalcin on Ca^{2+}/calmodulin-dependent phosphatase calcineurin activity toward phosphoserine or phosphothreonine. Calcineurin purified from rat brain was used in the enzyme assay. The reaction mixture contained either vehicle or regucalcin (0.12 μM) in the presence of both calcium chloride (10 μM) and calmodulin (2.0 μg/ml; 0.12 μM) with calcineurin (0.5 μg/ml). Each value represents the mean ± SEM of five experiments. *, $P < .01$, compared with the control value without calcium addition. Open bar: without Ca^{2+} addition, solid bar: with Ca^{2+} addition.

The reversibility of regucalcin effect on phosphatase calcineurine activity was examined (Fig. 6). Ca^{2+} (10 μM) plus calmodulin (0.12

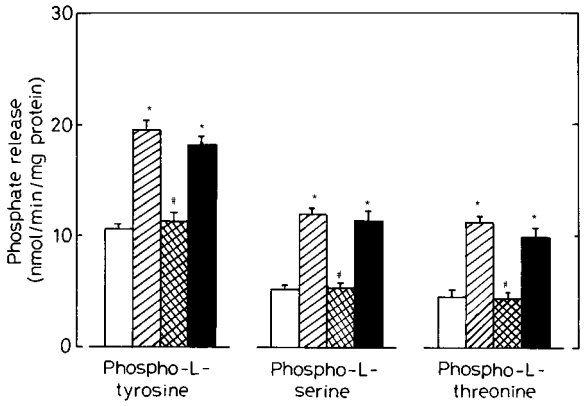

Fig. 6. Reversibility of regucalcin effect on Ca^{2+}/calmodulin-dependent phosphatase calcineurin activity toward phosphotyrosine, phosphoserine, and phosphothreonine. The enzyme reaction mixture contained either vehicle or regucalcin (0.12 μM) in the absence or presence of calcium chloride (10 μM) plus calmodulin (0.12 or 0.36 μM) with calcineurin (0.5 μg/ml). Each value represents the mean ± SEM of five experiments. *, $P < .01$, compared with the control value without calcium addition. #, $P < .01$, compared with the value obtained from Ca^{2+} and calmodulin addition without regucalcin. Open bar: Control, hatched bar: Ca^{2+} plus calmodulin addition, cross-hatched bar: Ca^{2+} plus calmodulin (0.12 μM) with regucalcin, solid bar: Ca^{2+} plus calmodulin (0.24 μM) with regucalcin.

μM) increased phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine as the substrate was completely prevented by the addition of regucalcin (0.12 μM). However, this inhibition was significantly reversed by the addition of the higher concentration of calmodulin (0.36 μM).

Binding of Regucalcin to Calmodulin

Whether regucalcin binds on calmodulin-agarose beads was examined, and the result is shown in Figure 7. The reaction mixture contained either vehicle (lane 1), regucalcin (lane 2; 10 μg/ml), calcium (lane 3; 0.2 mM), or regucalcin (10 μg/ml) plus calcium (0.2 mM) (lane 4) in the presence of calmodulin-agarose beads (containing 90 μg calmodulin). Incubated calmodulin-agarose beads were applied to SDS-PAGE. One band was found on SDS-PAGE. Regucalcin has been found to be 33,388 daltons [Shimokawa and Yamaguchi, 1993b]. This band on SDS-PAGE coincided with regucalcin used as a marker (lane 5). The binding of regucalcin to calmodulin was seen in both the absence and presence of Ca^{2+}.

Calcineurin has been demonstrated to bind to calmodulin [Tallent and Cheung, 1986]. Whether the binding of regucalcin on calmodulin-agarose beads is altered by the presence of calcineurin was examined (Fig. 8). The reaction mixture contained either vehicle (lane 1), regucalcin (lane 2; 10 μg/ml), calcineurin (lane 3; 2.5 μg/ml), or regucalcin (10 μg/ml) plus calcineurin (2.5 μg/ml) (lane 4) in the presence of calmodulin-agarose beads (containing 90 μg calmodulin). Calcineurin is a heterodimer, with subunit A of Mr 60,000 and submit B of Mr 19,000, present in a 1:1 ratio [Tallent and Cheung, 1986]. The band of calcineurin used as a marker (lane 6) was found at the position of about 70 kDa on SDS-PAGE. Both regucalcin and calcineurin were bound on calmodulin-agarose beads. However, regucalcin did not bind to calcineurin (data not shown).

DISCUSSION

Ca^{2+}-binding protein regucalcin has been shown to have an inhibitory effect on Ca^{2+}/calmodulin-dependent enzyme activity [Yamaguchi and Tai, 1991; Yamaguchi, 1992]. The regulatory mechanism of regucalcin to regulate calmodulin effect on enzyme activity, however, has not been fully clarified. Calcineurin, known

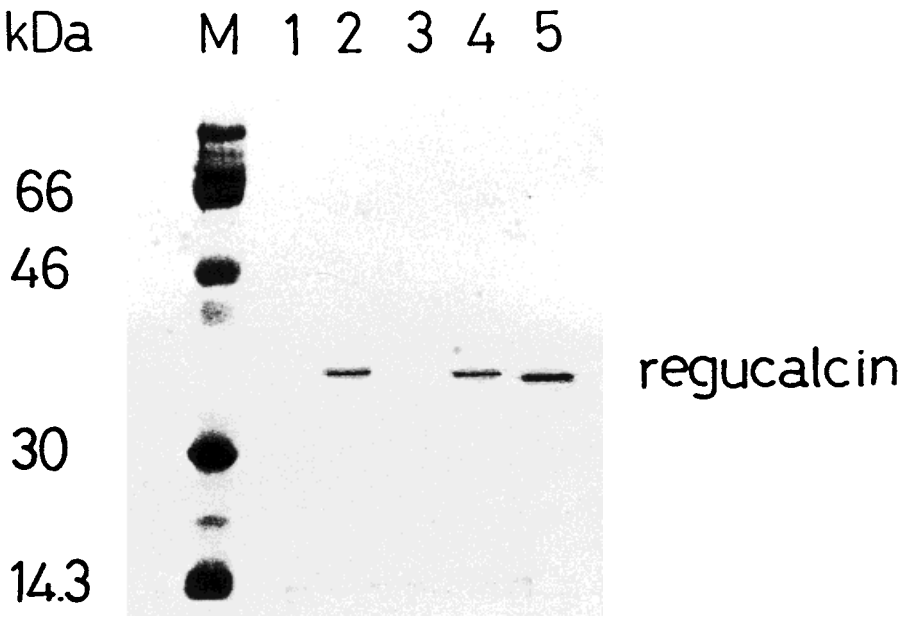

Fig. 7. Pattern of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of regucalcin bound to calmodulin-agarose beads. The reaction mixture contained either vehicle, regucalcin (10 μg/ml), calcium chloride (0.2 mM), or calcium chloride plus regucalcin (10 μg/ml) in the presence of calmodulin-agarose beads (90 μg calmodulin/ml), incubated for 60 min at 4°C. Samples for SDS-PAGE were pretreated with SDS in the presence of β-mercaptoethanol. The protein bands were visualized using Coomassie Blue staining. **Lane 1:** Control. **Lane 2:** Regucalcin. **Lane 3:** Calcium. **Lane 4:** Calcium plus regucalcin. **Lane 5:** Regucalcin (10 μg) as the marker. The result shows one of four experiments with separate samples.

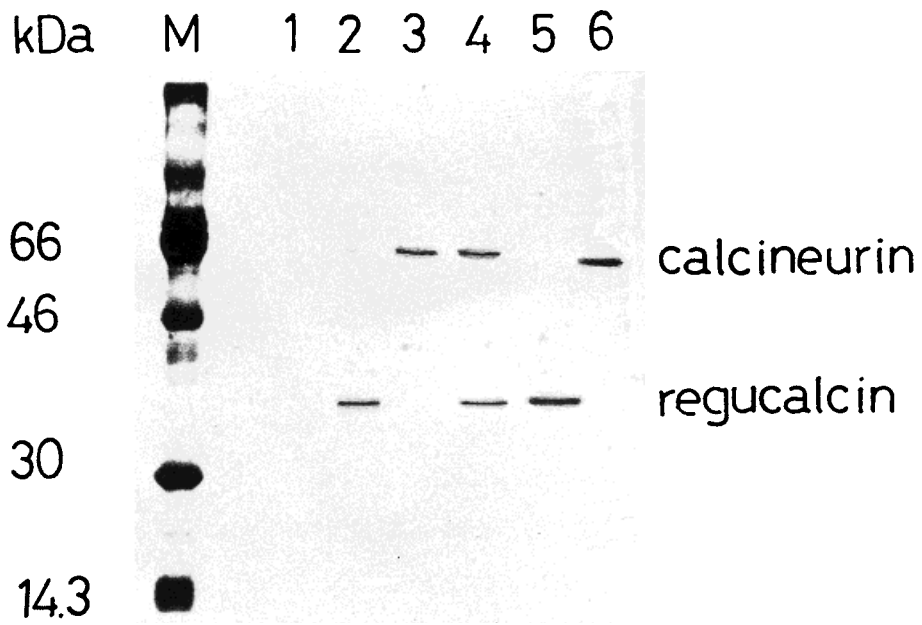

Fig. 8. Pattern of SDS-PAGE of regucalcin and calcineurin bound to calmodulin-agarose beads. The reaction mixture contained either vehicle, regucalcin (10 μg/ml), calcineurin (2.5 μg/ml), or regucalcin (10 μg/ml) plus calcineurin (2.5 μg/ml) in the presence of calmodulin-agarose beads (90 μg calmodulin/ml), incubated for 60 min at 4°C. Samples for SDS-PAGE was pretreated with SDS in the presence of β-mercaptoethanol. The protein bands were visualized using Coomassie Blue staining. **Lane 1:** Control. **Lane 2:** Regucalcin. **Lane 3:** Calcineurin. **Lane 4:** Regucalcin plus calcineurin. **Lane 5:** Regucalcin (10 μg) as the marker. **Lane 6:** Calcineurin (2.5 μg) as the marker. The result shows one of four experiments with separate samples.

as phosphatase 2B, is a Ca^{2+}- and calmodulin-dependent phosphatase [Tallent and Cheung, 1986; Wallace et al., 1980]. In mammals, calcineurin is most abundant in brain but also has been detected in liver cells [Tallent and Cheung, 1986; Wallace et al., 1980]. Calmodulin also exists in liver cells [Kakiuchi et al., 1982]. Whether regucalcin can regulate Ca^{2+}/calmodulin-dependent protein phosphatase activity in liver cells has not been reported so far.

Regucalcin is mainly localized in the cytoplasm of rat liver [Shimokawa and Yamaguchi, 1993a; Yamaguchi and Isogai, 1993]. Whether exogenous regucalcin has an inhibitory effect on Ca^{2+}/calmodulin-dependent phosphatase activity in rat liver cytosol was examined. The addition of Ca^{2+} and calmodulin in the enzyme reaction mixture using liver cytosol caused a significant increase in phosphatase activity toward phosphamino acids (including phosphotyrosine, phosphoserine, and phosphothreonine) in liver cytosol, indicating that Ca^{2+}/calmodulin-dependent protein phosphatase activity exists in liver cytosol. This increase was completely inhibited by the addition of regucalcin with the higher concentrations than that of endogenous regucalcin, which existed in the reaction mixture using liver cytosolic protein. This result suggests that regucalcin has an inhibitory effect on Ca^{2+}/calmodulin-dependent phosphatase activity in liver cytosol.

The inhibitory role of endogenous regucalcin on Ca^{2+}/calmodulin-dependent phosphatase activity in rat liver cytosol, moreover, was examined using anti-regucalcin antibody. The presence of anti-regucalcin antibody in the enzyme reaction mixture caused a significant elevation of phosphoamino acid phosphatase activity in liver cytosol. Such an effect was not seen in the presence of mouse IgG or in the combination with exogenous regucalcin. These results suggest that endogenous regucalcin plays an inhibitory role in the regulation of Ca^{2+}/calmodulin-dependent protein phosphatase activity in the cytoplasm of liver cells. Rat liver contains about 6.0 μM calmodulin and about 48 μM regucalcin [Yamaguchi and Isogai, 1993]. Thus, regucalcin was more abundant than calmodulin in liver tissues. Presumably, regucalcin has a physiological role in the process mediated through the activation of Ca^{2+}/calmodulin-dependent phosphatase in liver cells.

Regucalcin completely inhibited Ca^{2+}/calmodulin-dependent phosphatase activity using puri-

fied calcineurin. This inhibitin, however, was reversed by the addition of higher concentrations of calmodulin. Regucalcin was bound on calmodulin-agarose beads as analyzed by SDS-PAGE, suggesting that regucalcin is a calmodulin-binding protein. Regucalcin was also bound on calmodulin-agarose beads in the presence of calcineurin, which can bind to calmodulin [Tallent and Cheung, 1986]. These findings suggest the possibility that regucalcin inhibits Ca^{2+}/calmodulin-dependent phosphatase calcineurin activity by regucalcin binding to calmodulin. Presumably, the binding of regucalcin to calmodulin changes the conformation of calmodulin-binding calcineurin. The inhibitory mechanism of regucalcin, however, remains to be elucidated.

The role of Ca^{2+} in the hormonal regulation of liver metabolism has been demonstrated [Bygrave and Benedetti, 1993; Williamson et al., 1981]. Calmodulin, which can amplify Ca^{2+} action, plays a pivotal role in cellular regulation [Cheung, 1980]. Regucalcin has been shown to have an inhibitory effect on Ca^{2+}/calmodulin-dependent enzyme activity [Yamaguchi and Tai, 1991; Yamaguchi, 1992]. In the current study, regucalcin binds to calmodulin and inhibits Ca^{2+}/calmodulin-dependent protein phosphatase activity in liver cells. It is proposed that regucalcin may be having a general inhibitory role in all Ca^{2+}/calmodulin-dependent processes in liver cells.

At present, a physiological role of regucalcin action on Ca^{2+}/calmodulin-dependent protein phosphatase activity in liver cells is unknown. There may be many proteins phosphorylated by protein kinases in liver cells. Phosphorylated proteins are dephosphorylated by protein phosphatases. Regucalcin may have a role in the regulation of signaling processes, which are mediated through Ca^{2+}/calmodulin-dependent protein phosphatase in liver cells. Meanwhile, regucalcin has been shown to inhibit Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C activities in rat liver cytosol [Yamaguchi, 1992; Yamaguchi and Tai, 1991]. Presumably, regucalcin plays a physiological role in the intracellular control of the hormonal stimulation for phosphorylation and dephosphorylation of many proteins in liver cells.

In conclusion, it has been demonstrated that regucalcin inhibits Ca^{2+}/calmodulin-dependent phosphatase activity in liver cells and that regucalcin can bind to calmodulin.

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