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Stimulatory Effect of Regucalcin on ATP-Dependent Ca^{2+} Uptake Activity in Rat Liver Mitochondria

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Abstract The effect of Ca^{2+}-binding protein regucalcin on Ca^{2+}-ATPase activity in isolated rat liver mitochondria was investigated. The presence of regucalcin (0.1, 0.25, and 0.5 μM) in the enzyme reaction mixture led to a significant increase in Ca^{2+}-ATPase activity. Regucalcin significantly stimulated ATP-dependent $^{45}Ca^{2+}$ uptake by the mitochondria. Ruthenium red (10^{-5} M) or lanthanum chloride (10^{-4} M), an inhibitor of mitochondrial Ca^{2+} uptake, completely inhibited regucalcin (0.25 μM)-increased mitochondrial Ca^{2+}-ATPase activity and $^{45}Ca^{2+}$ uptake. The effect of regucalcin (0.25 μM) in increasing Ca^{2+}-ATPase activity was completely inhibited by the presence of digitonin (10^{-2}%), a solubilizing reagent of membranous lipids, or vanadate (10^{-5} M), an inhibitor of phosphorylation of ATPase. The activatory effect of regucalcin (0.25 μM) on Ca^{2+}-ATPase activity was not further enhanced in the presence of dithiothreitol (2.5 mM), a protecting reagent of the sulfhydryl (SH) group of the enzyme, or calmodulin (0.60 μM), a modulator protein of Ca^{2+} action that could increase mitochondrial Ca^{2+}-ATPase activity. The present study demonstrates that regucalcin can stimulate Ca^{2+} pump activity in rat liver mitochondria, and that the protein may act on an active site (SH group)-related to phosphorylation of mitochondrial Ca^{2+}-ATPase. J. Cell. Biochem. 78: 121–130, 2000.

Key words: regucalcin; Ca^{2+} transport; Ca^{2+}-ATPase; mitochondria; rat liver

The calcium ion (Ca^{2+}) plays a role as an important second messenger signal in a variety of pathways to produce a Ca^{2+}-mediated physiologic responses in many cells. The Ca^{2+} signal is transmitted to an intracellular response partly via a family of Ca^{2+}-binding proteins [Cheung, 1980; Heizmann and Hunziker, 1991; Bygrave and Benedetti, 1993]. Regucalcin is a novel Ca^{2+}-binding protein that exists in the cytoplasm of rat liver and kidney [Yamaguchi, 1992, 2000; Shimokawa and Yamaguchi, 1992]. The regucalcin gene is localized on the proximal end of rat chromosome Xq11.1-12 [Shimokawa et al., 1995]. The rat regucalcin gene consists of seven exons and six introns [Yamaguchi et al., 1996]. Promotor activity of the regucalcin gene in liver cells has been shown to be stimulated by the Ca^{2+} signal [Murata and Yamaguchi, 1999]. The hepatic regucalcin mRNA expression has been promoted by calcium administration to rats [Shimokawa and Yamaguchi, 1992]. Thus, the expression of regucalcin may be stimulated by an increase in intracellular Ca^{2+} level in liver cells.

Regucalcin has been proposed to play an important role in the regulation of Ca^{2+} signaling in liver and kidney cells; the protein has an inhibitory effect on Ca^{2+}/calmodulin-dependent enzyme activation, protein kinase C activation, and Ca^{2+}-activated DNA fragmentation [Yamaguchi and Sakurai, 1991; Yamaguchi and Katsumata, 1999; Yamaguchi, 2000]. Regucalcin may be a regulatory protein in cellular function related to Ca^{2+} in liver and kidney cells.

The regulation of intracellular Ca^{2+} homeostasis is implicated in cellular functions. The low cytoplasmic Ca^{2+} concentration of living cells is maintained by energy-requiring pumps. These pumps either remove Ca^{2+} to the extracellular space by transport across the plasma membrane or accumulate it inside intracellular organelles such as the mitochondria and endoplasmic reticulum [Carafoli and Zurini, 1982; Nicholls and Akerman, 1982;

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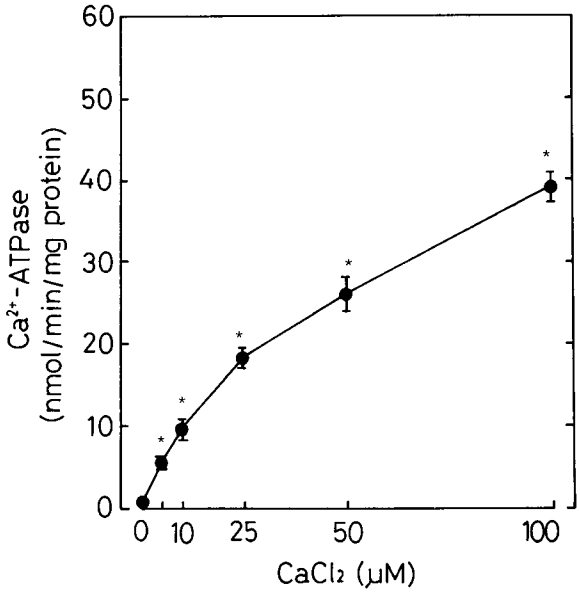

Fig. 1. Alteration in Ca^{2+}-ATPase activity with increasing concentrations of Ca^{2+} addition in rat liver mitochondria. $CaCl_2$ was added to the enzyme reaction mixture, yielding concentrations of 5, 10, 25, 50, and 100 μM. Each value is the mean ± SEM of five experiments with separate rats.
*$P < 0.01$, as compared with the control value without Ca^{2+} addition.

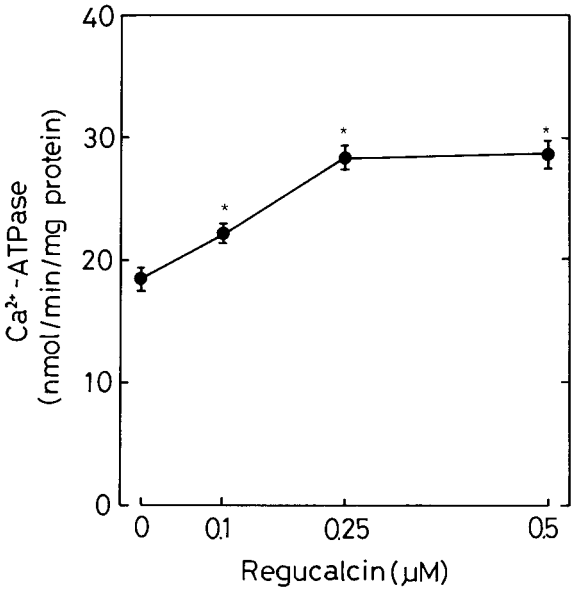

Fig. 2. Effect of regucalcin on Ca^{2+}-ATPase activity in rat liver mitochondria. Regucalcin was added to the enzyme reaction mixture, yielding concentrations of 0.1, 0.25, and 0.5 μM in the presence of 50 μM $CaCl_2$. Each value is the mean ± SEM of five experiments with separate rats.
*$P < 0.01$, as compared with the control value without regucalcin addition.

Kraus-Friedmann, 1990]. Regucalcin has been shown to stimulate Ca^{2+} pump activity in rat liver plasma membranes [Takahashi and Yamaguchi, 1994, 1997] and microsomes [Takahashi and Yamaguchi, 1999], suggesting that the protein plays a role in the regulation of intracellular Ca^{2+} homeostasis. The role of regucalcin in the regulation of mitochondrial Ca^{2+} sequestration in liver cells, however, has not been fully clarified.

The present study was therefore undertaken to determine the effect of regucalcin on Ca^{2+}-ATPase activity, which is related to ATP-dependent Ca^{2+} uptake by liver mitochondria. We found that regucalcin has a stimulatory effect on ATP-dependent Ca^{2+} uptake activity in rat liver mitochondria.

MATERIALS AND METHODS

Chemicals

Adenosine-5′-triphosphate (ATP), ruthenium red, lanthanum chloride ($LaCl_3$), dithiothreitol, digitonin, and calmodulin (56,500 units/mg protein from bovine brain) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{45}Ca]-calcium chloride (12.4 GBq/mg) was obtained from New England Nuclear (Boston, MA). Calcium chloride, vanadate, and all other chemicals were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Many 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 Japan SLC (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, 1992, 1993]. 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, suspended 1:4 (wt/vol) in Tris-HCl buffer (pH 7.4), and

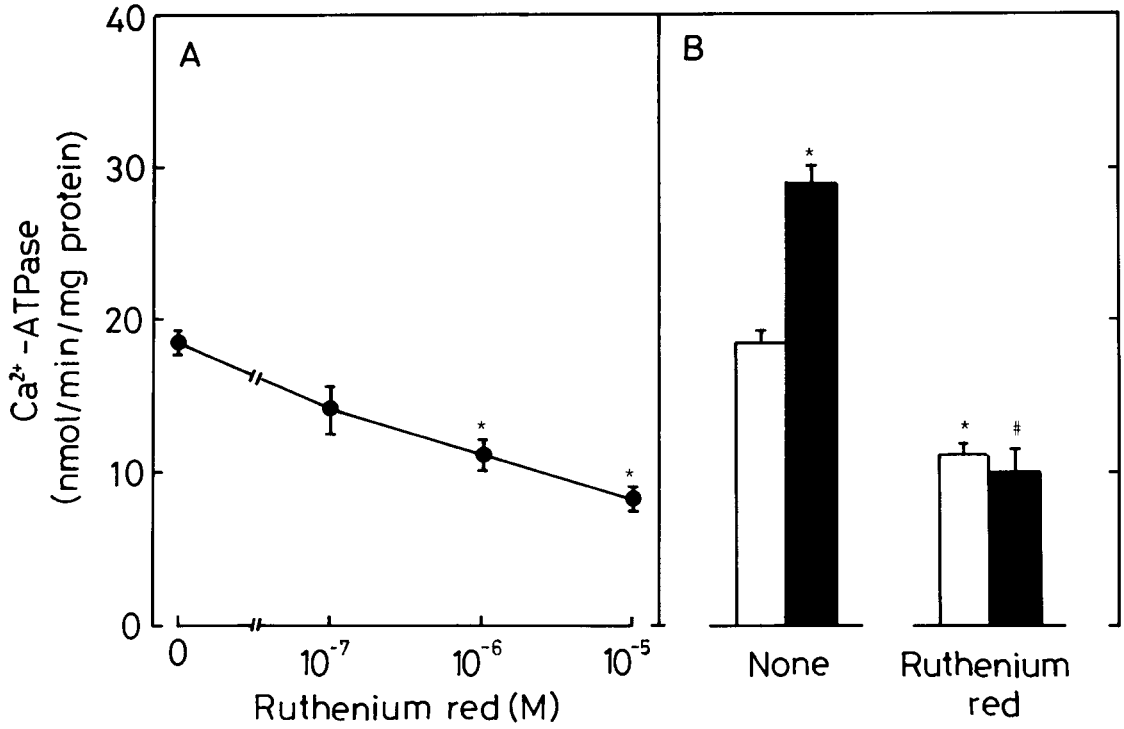

Fig. 3. Effect of ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, on the regucalcin-increased Ca^{2+}-ATPase activity in rat liver mitochondria. **A:** Ruthenium red was added to the enzyme reaction mixture, yielding concentrations of 10^{-7}–10^{-5} M in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (0.25 μM) in the absence or presence of ruthenium red (10^{-6} M). Each value is the mean ± SEM of five experiments with separate rats. Open bar, control; closed bar, regucalcin.
*$P < 0.01$, as compared with the control (none) value.
#$P < 0.01$, as compared with the value of regucalcin alone.

homogenized in a Potter-Elvehjem homogenenizer with a Teflon pestle. The homogenate was spun at 5,500g in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000g for 60 min. The resulting supernatant was heated at 60°C for 10 min and recentrifuged at 38,000g 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].

Preparation of Liver Mitochondria

Rats were killed by cardiac puncture, and the liver was perfused with ice-cold 250 mM sucrose solution, immediately cut into small pieces, suspended 1:9 in the homogenization medium containing 250 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. (HEPES), and 1.0 mM ethyleneglycol bis(2-amino-ethylether)-N,N,N′,N′,-tetraacetic acid (EGTA), pH 7.4, and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle [Berthon et al., 1981]. The homogenate was centrifuged at 800g for 10 min to remove nuclei, unbroken cells, and cell debris. The resultant supernatant was centrifuged at 8,500g for 10 min to sediment the mitochondrial fraction. The mitochondrial fraction was resuspended in 5 mM $MgCl_2$, 50 mM KCl, and 10 mM HEPES, pH 7.0, to a final protein concentration of 1.8–2.5 mg/ml.

Assay of Ca^{2+}-ATPase

Mg^{2+}-ATPase activity was determined for 10 min at 37°C in a medium containing 10 mM HEPES-KOH buffer (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 6 mM succinate, 8 mM Mg-ATP, and the mitochondria (180–250 μg as protein) in the absence or presence of regucalcin (0.1–0.5 μM) [Vale et al., 1983]. The amount of inorganic phosphate released from ATP by enzyme reaction was measured according to the method of Nakamura and Mori [1958]. (Ca^{2+} +

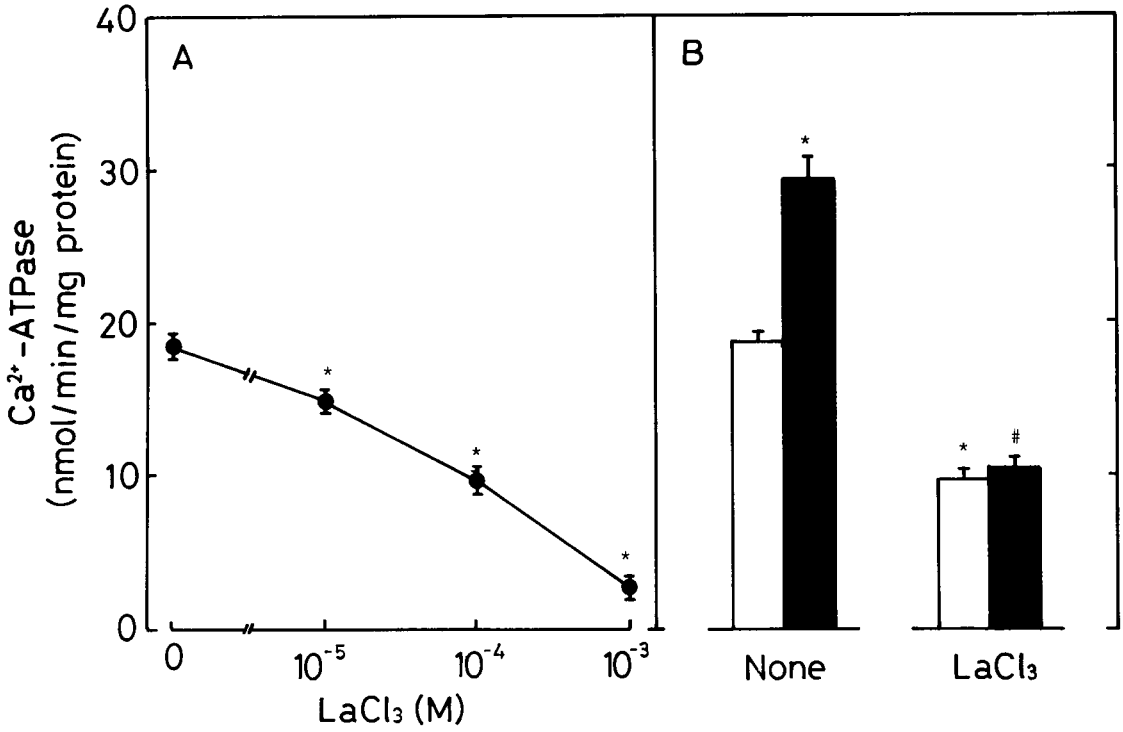

Fig. 4. Effect of lanthanum chloride ($LaCl_3$) on the regucalcin-increased Ca^{2+}-ATPase activity in rat liver mitochondria. **A:** $LaCl_3$ was added to the enzyme reaction mixture, yielding concentrations of 10^{-5}–10^{-3} M in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (0.25 μM) in the absence or presence of $LaCl_3$ (10^{-4} M). Each value is the mean ± SEM of five experiments with separate rats. Open bar, control; closed bar, regucalcin. *$P < 0.01$, as compared with the control (none) value. #$P < 0.01$, as compared with the value of regucalcin alone.

Mg^{2+})-ATPase activity was measured in the same medium, but with Tris-EGTA replaced by 50 μM $CaCl_2$ in the absence or presence of regucalcin (0.1–0.5 μM). Ca^{2+}-ATPase activity was calculated as the difference between (Ca^{2+} + Mg^{2+})-ATPase and Mg^{2+}-ATPase. Enzyme activity was expressed as nmol of inorganic phosphate released per minute per milligram protein. Protein concentration was determined by the method of Lowry et al. [1951].

ATP-Dependent $^{45}Ca^{2+}$ Uptake

$^{45}Ca^{2+}$ uptake was measured by the Millipore filtration technique [Whiting and Barritt, 1982]. The mitochondria (180–250 μg of protein/ml of reaction mixture) was preincubated for 1 min at 37°C in 1 ml of medium containing 150 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 4 μM rotenone, and 50 μM $CaCl_2$ containing $^{45}Ca^{2+}$ (0.185 MBq), pH 7.4, in the absence or presence of regucalcin (0.1–0.5 μM). At 10 min after the addition of 2 mM ATP, adjusted to pH 7.4 with KOH, to initiate energy-dependent Ca^{2+} uptake, a 0.5-ml sample was filtered through a 0.22-μm prewetted Millipore filter. The precipitate was washed with 150 mM KCl/10 mM HEPES, pH 7.4, transferred to a scintillation vial, and counted for radioactivity. $^{45}Ca^{2+}$ uptake is expressed as nanomoles of $^{45}Ca^{2+}$ accumulated per milligram protein of the mitochondria.

Statistical Analysis

Data were expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. A *P*-value of 0.05 was considered to indicate a statistically significant difference.

RESULTS

Effect of Regucalcin on Liver Mitochondrial Ca^{2+}-ATPase Activity

The effect of calcium chloride addition on Ca^{2+}-ATPase activity in rat liver mitochondria

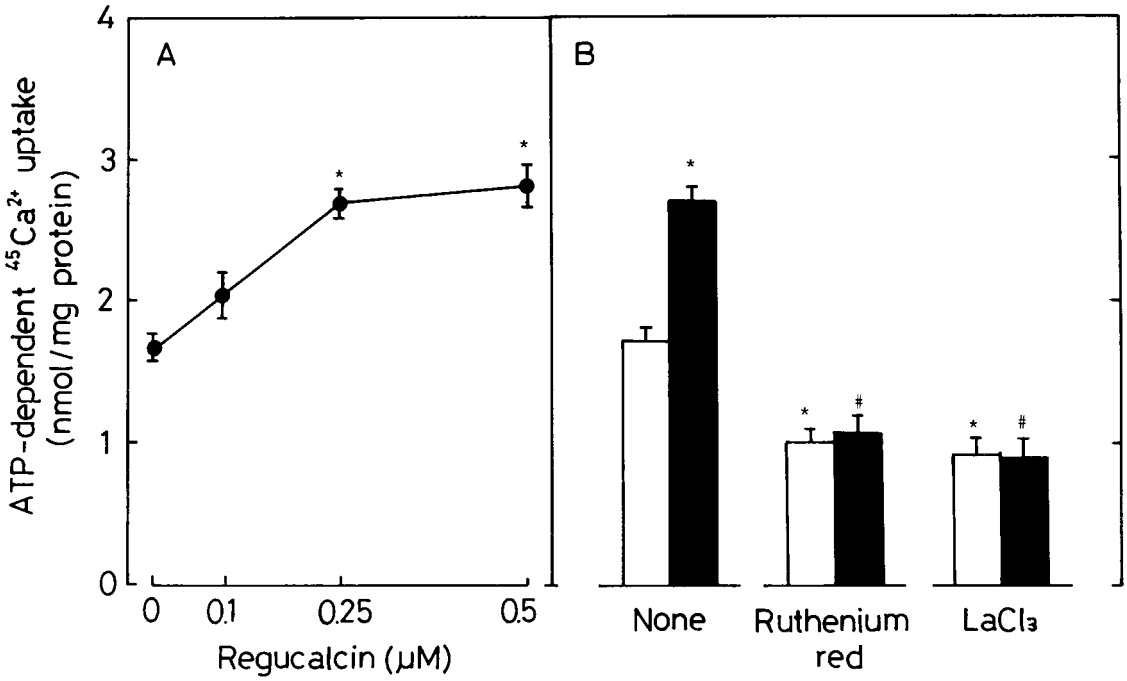

Fig. 5. Effect of regucalcin on the ATP-dependent $^{45}Ca^{2+}$ uptake in rat liver mitochondria. $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods. **A:** The mitochondria were incubated for 10 min after the addition of ATP in the absence or presence of regucalcin (0.1, 0.25, and 0.5 μM). **B:** The mitochondria were incubated for 10 min after the addition of ATP in a reaction mixture containing either vehicle, ruthenium red (10^{-6} M), or $LaCl_3$ (10^{-4} M) in the absence or presence of regucalcin (0.25 μM). Each value is the mean ± SEM of five experiments with separate rats. Open bar, control; closed bar, regucalcin.
*$P < 0.01$, as compared with the control value without regucalcin addition.
#$P < 0.01$, as compared with the value of regucalcin alone.

is shown in Fig. 1. The addition of calcium chloride (5, 10, 25, 50, and 100 μM) in the enzyme reaction mixture led to a significant increase in Ca^{2+}-ATPase activity. In the presence of 50 μM $CaCl_2$, the addition of regucalcin (0.1, 0.25, and 0.5 μM) produced a significant elevation of Ca^{2+}-ATPase activity. The effect of regucalcin reached a maximum at the concentration of 0.25 μM (Fig. 2). Meanwhile, liver mitochondrial Mg^{2+}-ATPase activity in the presence of 2 mM EGTA was not appreciably altered by the addition of regucalcin (0.1–0.5 μM) (data not shown). Ruthenium red or lanthanum chloride ($LaCl_3$) is an inhibitor of the mitochondrial Ca^{2+} uniporter [Nicholls and Akerman, 1982]. Hepatic mitochondrial Ca^{2+}-ATPase activity was markedly decreased by the addition of ruthenium red (10^{-6} and 10^{-5} M) (Fig. 3A) or $LaCl_3$ (10^{-5}–10^{-3} M) (Fig. 4A). The effect of regucalcin (0.25 μM) in increasing Ca^{2+}-ATPase activity was not entirely seen in the presence of ruthenium red (10^{-6} M) or $LaCl_3$ (10^{-4} M) (Fig. 3B and 4B), indicating that regucalcin acts on ruthenium red- or

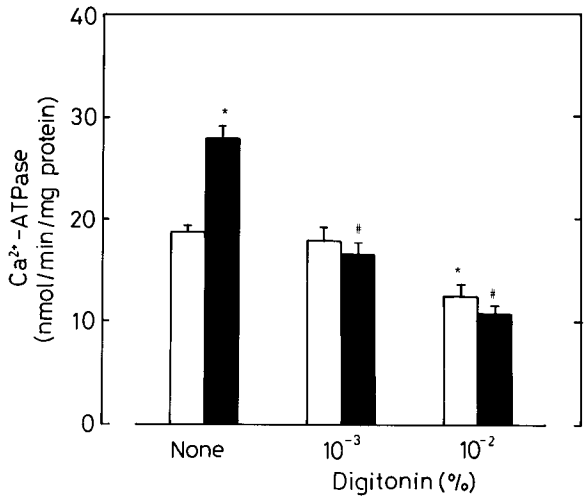

Fig. 6. Effect of digitonin on the regucalcin-increased Ca^{2+}-ATPase activity in rat liver mitochondria. The enzyme reaction mixture contained either vehicle or digitonin (10^{-3}% or 10^{-2}%) in the absence or presence of regucalcin (0.25 μM) with 50 μM $CaCl_2$. Each value is the mean ± SEM of five experiments with separate rats. Open bar, control; closed bar, regucalcin.
*$P < 0.01$, as compared with the control (none) value.
#$P < 0.01$, as compared with the value of regucalcin alone.

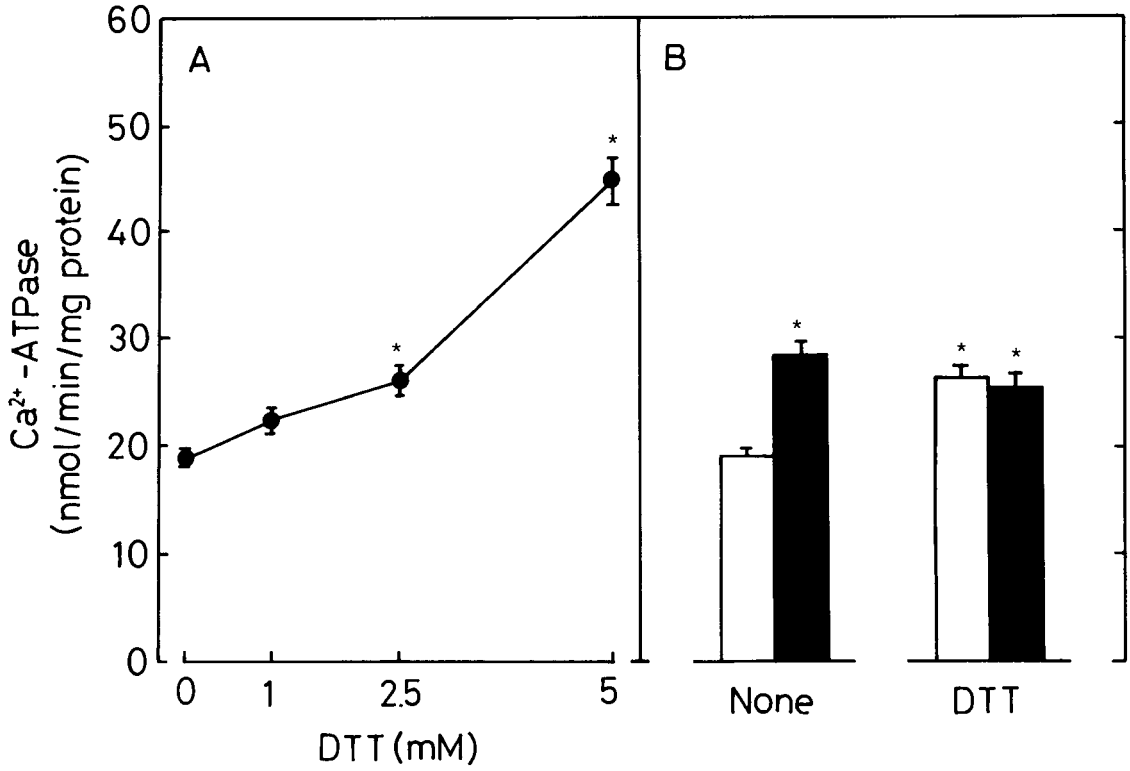

Fig. 7. Effect of dithiothreitol (DTT) on the regucalcin-increased Ca^{2+}-ATPase activity in rat liver mitochondria. **A:** DTT was added to the enzyme reaction mixture, yielding concentrations of 1, 2.5, and 5.0 mM in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction contained either vehicle or regucalcin (0.25 μM) in the absence or presence of DTT (2.5 mM). Each value is the mean ± SEM of five experiments with separate rats. Open bar, control; closed bar, regucalcin.
*$P < 0.01$, as compared with the control (none) value.
#$P < 0.01$, as compared with the value of regucalcin alone.

$LaCl_3$-sensitive Ca^{2+}-ATPase (uniporter) in the mitochondria.

The effect of regucalcin on ATPase-dependent $^{45}Ca^{2+}$ uptake by liver mitochondria is shown in Fig. 5. The addition of ATP to the reaction mixture containing $^{45}Ca^{2+}$ led to mitochondrial $^{45}Ca^{2+}$ uptake (Fig. 5A). This uptake was significantly increased by the presence of regucalcin (0.25 and 0.5 μM) (Fig. 5A). The effect of regucalcin (0.25 μM) in elevating mitochondrial $^{45}Ca^{2+}$ uptake was completely prevented in the presence of ruthenium red (10^{-6} M) or $LaCl_3$ (10^{-4} M) (Fig. 5B). Ruthenium red (10^{-6} M) or $LaCl_3$ (10^{-4} M) alone had a significant inhibitory effect on mitochondrial $^{45}Ca^{2+}$ uptake (Fig. 5B). Thus, regucalcin reveals an activatory effect on liver mitochondrial Ca^{2+} uptake activity.

Characterization of Regucalcin Action on Liver Mitochondrial Ca^{2+}-ATPase Activity

The effect of digitonin on the regucalcin-increased Ca^{2+}-ATPase activity in hepatic mitochondria is shown in Fig. 6. Digitonin has a solubilization effect on membranous lipids [Murphy et al., 1980]. The presence of digitonin (10^{-2}%) in the enzyme reaction mixture caused a significant decrease in mitochondrial Ca^{2+}-ATPase activity. In the presence of digitonin (10^{-3}% or 10^{-2}%), regucalcin (0.25 μM) could not elevate Ca^{2+}-ATPase activity.

The effect of dithiothreitol (DTT), a protecting reagent of sulfhydryl (SH) groups, on the regucalcin-increased Ca^{2+}-ATPase activity in liver mitochondria is shown in Fig. 7. The presence of DTT (2.5 and 5.0 mM) in the enzyme reaction mixture caused a remarkable elevation in Ca^{2+}-ATPase activity (Fig. 7A). In the presence of DTT (2.5 mM), the effect of regucalcin (0.25 μM) in increasing Ca^{2+}-ATPase activity was not further enhanced (Fig. 7B).

The effect of vanadate, an inhibitor of Ca^{2+}-dependent phosphorylation of (Ca^{2+} − Mg^{2+})-ATPase in rat liver plasma membranes [Chen and Junger, 1983], on the regucalcin-increased Ca^{2+}-ATPase activity in liver mitochondria is

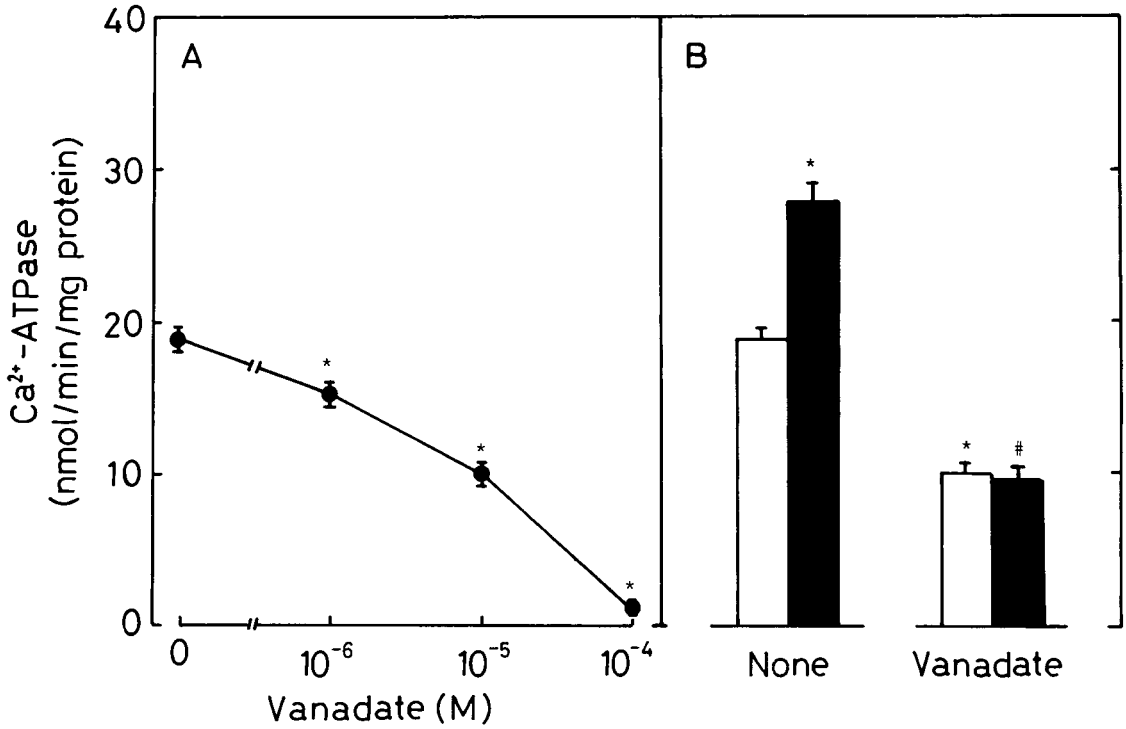

Fig. 8. Effect of vanadate on the regucalcin-increased Ca^{2+}-ATPase activity in rat liver mitochondria. **A:** Vanadate was added to the enzyme reaction mixture, yielding concentrations of 10^{-6}–10^{-4} M in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (0.25 μM) in the absence or presence of vanadate (10^{-5} M). Each value is the mean ± SEM of five experiments with separate rats. Open bar, control; closed bar, regucalcin.
*$P < 0.01$, as compared with the control (none) value.
#$P < 0.01$, as compared with the value of regucalcin alone.

shown in Fig. 8. The presence of vanadate (10^{-6}–10^{-4} M) led to a significant decrease in Ca^{2+}-ATPase activity (Fig. 8A). The effect of regucalcin (0.25 μM) in elevating Ca^{2+}-ATPase activity was not seen in the presence of vanadate (10^{-5} M) (Fig. 8B).

The effect of calmodulin, which can modulate Ca^{2+} action [Cheung, 1980], on the regucalcin-increased Ca^{2+}-ATPase activity in liver mitochondria is shown in Fig. 9. Mitochondrial Ca^{2+}-ATPase activity was significantly raised by the presence of calmodulin (1, 5, and 10 μg/ml; 0.06–0.6 μM) in the enzyme reaction mixture (Fig. 9A). The effect of calmodulin was saturated at 5 μg/ml. The effect of regucalcin in increasing Ca^{2+}-ATPase activity was not further enhanced by the presence of calmodulin (10 μg/ml) (Fig. 9B).

DISCUSSION

Regucalcin has been shown to activate Ca^{2+}-pump enzymes in the plasma membranes and microsomes isolated from rat liver [Takahashi and Yamaguchi, 1994, 1997, 1999], suggesting that the protein plays a role in the regulation of intracellular Ca^{2+} homeostasis. In the present study, regucalcin has been found to increase Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake in liver mitochondria. Thus, regucalcin may be a physiologic role as an activatory protein in the regulation of Ca^{2+}-pump–related enzymes in liver cells.

A Ca^{2+} carrier of the mitochondria has been reported to be a Ca^{2+}-binding glycoprotein [Panfili et al., 1980], but the energy of respiration or of ATP hydrolysis is utilized to generate a membrane potential (and a proton gradient) that is responsible for driving Ca^{2+} electrophoretically [Brand and Lehninger, 1975; Nicholls, 1978]. The present study clearly demonstrates that regucalcin increases Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake in liver mitochondria, and that these increases are completely blocked by ruthenium red or lanthanum chloride, which are specific inhibitors of the Ca^{2+} uniporter in the mitochondria [Nicholls and Akerman, 1982]. This finding suggests that regucalcin stimulates Ca^{2+}-

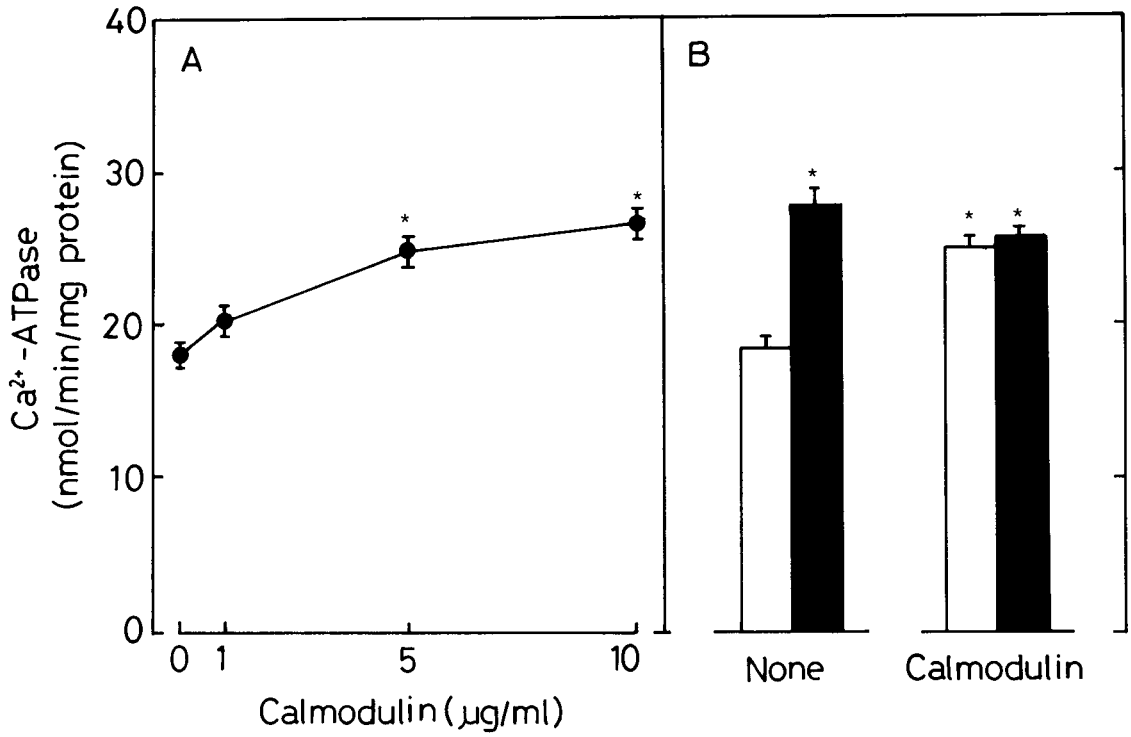

Fig. 9. Effect of calmodulin on the regucalcin-increased Ca^{2+}-ATPase activity in rat liver mitochondria. **A:** Calmodulin was added to the enzyme reaction mixture, yielding concentrations of 1, 5, and 10 μg/ml in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction contained either vehicle or regucalcin (0.25 μM) in the absence or presence of calmodulin (10 μg/ml; 0.6 μM). Each value is the mean ± SEM of five experiments with separate rats. Open bar, control; closed bar, regucalcin.
*$P < 0.01$, as compared with the control (none) value.
#$P < 0.01$, as compared with the value of regucalcin alone.

ATPase–related Ca^{2+} uniporter activity in liver mitochondria.

The molecular mechanism of regucalcin action on hepatic mitochondrial $^{45}Ca^{2+}$ uptake is unknown. However, the effect of regucalcin in increasing mitochondrial Ca^{2+}-ATPase activity was characterized. We found that the regucalcin-increased mitochondrial Ca^{2+}-ATPase activity is inhibited by the presence of digitonin, a solubilization reagent of membranous lipids [Murphy et al., 1980], in the enzyme reaction mixture. This result suggests that regucalcin acts on Ca^{2+}-ATPase by its bonding on the mitochondrial membranous lipids. In fact, it has been shown that radioiodinated regucalcin can bind to isolated rat liver mitochondria [Yamaguchi et al., 1988]. Moreover, the stimulatory effect of regucalcin on mitochindrial Ca^{2+}-ATPase activity was not further enhanced by the presence of DTT, a protecting reagent of SH groups, which could increase the enzyme activity. It has been reported that liver microsomal Ca^{2+} sequestration is critically dependent on the SH groups of protein [Thor et al., 1985], and that regucalcin can increase Ca^{2+}-ATPase activity acting on the SH groups, which may be an active site of the enzyme in hepatic microsomes [Takahashi and Yamaguchi, 1999]. On the basis of our results, it is assumed that regucalcin acts on the SH groups of Ca^{2+}-ATPase binding to the membranous lipids of liver mitochondria.

Vanadate has been shown to be an inhibitor of Ca^{2+}-dependent phosphorylation of (Ca^{2+} – Mg^{2+})-ATPase in rat liver plasma membranes [Chen and Junger, 1983]. Vanadate could inhibit Ca^{2+}-ATPase activity in hepatic mitochondria, and it caused a complete inhibition of the regucalcin-increased enzyme activity. From this finding, it appears that regucalcin can stimulate Ca^{2+}-dependent phosphorylation of Ca^{2+}-ATPase in liver mitochondria.

Calmodulin has been reported to be present in liver mitochondria [Hatase et al., 1985; Gazzotti et al., 1984]. We find here that calmodulin increases Ca^{2+}-ATPase activity in rat liver mitochondria, suggesting that calmodulin can stimulate Ca^{2+} uptake by the mitochondria.

The effect of regucalcin, however, was not further enhanced by the presence of calmodulin. Regucalcin and calmodulin may regulate reciprocally Ca^{2+} uptake activity in the mitochondria of liver cells. Meanwhile, regucalcin has an activatory effect on the Ca^{2+}-pump enzyme [$(Ca^{2+} - Mg^{2+})$-ATPase] in liver plasma membranes (Takahashi and Yamaguchi, 1994, 1997), although calmodulin does not have an effect on the enzyme activity (Lotersztajn et al., 1981). In addition, the preparation of regucalcin did not contain calmodulin by estimating with Western blot analysis, indicating that the activatory effect of regucalcin on mitochondrial Ca^{2+}-ATPase is not resulted from calmodulin.

The low cytoplasmic Ca^{2+} concentration of living cells is maintained by energy-requiring pumps. These pumps either remove Ca^{2+} to the extracellular space by transport across the plasma membrane or accumulate it inside intracellular organelles such as the mitochondria and endoplasmic reticulum (microsomes). Regucalcin could stimulate the plasma membrane $(Ca^{2+} - Mg^{2+})$-ATPase activity [Yamaguchi et al., 1988; Takahashi and Yamaguchi, 1994, 1997], the microsomal Ca^{2+}-ATPase activity [Takahashi and Yamaguchi, 1999], and the mitochondrial ATP-dependent Ca^{2+} uptake activity in liver cells. Regucalcin is a Ca^{2+}-binding protein isolated from rat liver cytoplasm [Yamaguchi and Yamamoto, 1978], and the metal from $^{45}Ca^{2+}$-binding regucalcin is transported to liver mitochondria and microsomes using the energy of ATP hydrolysis [Yamaguchi, 1985]. Regucalcin may play a physiologic role as an activator protein of Ca^{2+}-pump enzymes in the regulation of intracellular Ca^{2+} homeostasis in liver cells.

In conclusion, it has been demonstrated that regucalcin has a stimulatory effect on Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake in isolated rat liver mitochondria.

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