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Stimulatory Effect of Regucalcin on Mitochondrial ATP-Dependent Calcium Uptake Activity in Rat Kidney Cortex

Jing Hui Xue, Hiroko Takahashi, and Masayoshi Yamaguchi*

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, Shizuoka, Japan

Abstract The effect of regucalcin, which is a regulatory protein of Ca^{2+} signaling, on Ca^{2+}-ATPase activity in isolated rat renal cortex mitochondria was investigated. The presence of regucalcin (50, 100, and 250 nM) 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^{-6} M) or lanthunum chloride (10^{-6} M), an inhibitor of mitochondrial Ca^{2+} uptake, markedly inhibited regucalcin (100 nM)-increased mitochondrial Ca^{2+}-ATPase activity and $^{45}Ca^{2+}$ uptake. The effect of regucalcin (100 nM) in elevating Ca^{2+}-ATPase activity was completely prevented by the presence of digitonin (10^{-2}%), a solubilizing reagent of membranous lipids, vanadate, an inhibitor of phosphorylation of ATPase, or dithiothreitol (50 mM), a protecting reagent of the sulfhydryl (SH) group of the enzyme. The activating effect of regucalcin (100 nM) on Ca^{2+}-ATPase activity was not further enhanced by calmodulin (0.30 μM) or dibutyryl cyclic AMP (10^{-4} M), which could increase Ca^{2+}-ATPase activity. Trifluoperazine (TFP; 50 μM), an antagonist of calmodulin, significantly decreased Ca^{2+}-ATPase activity. The activating effect of regucalcin on the enzyme was also seen in the presence of TFP, indicating that regucalcin's effect is not involved in mitochondrial calmodulin. The present study demonstrates that regucalcin can stimulate Ca^{2+}-pump activity in rat renal cortex mitochondria, and that the protein may act on an active site (SH group) related to phosphorylation of mitochondrial Ca^{2+}-ATPase. J. Cell. Biochem. 80:285–292, 2000.

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

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. The Ca^{2+} signal in cells for hormonal stimulation is partly transmitted to intracellular responses, which are mediated through a family of Ca^{2+}-binding proteins [Cheung, 1980; Heizmann and Hunziker, 1991; Kraus-Friedman, 1990]. Regucalcin is a novel Ca^{2+}-binding protein that does not contain the EF-hand motif as a Ca^{2+}-binding domain [Yamaguchi and Yamamoto, 1978; Shimokawa and Yamaguchi, 1993]. In recent years, regucalcin has been demonstrated to play a role as a regulatory protein of Ca^{2+} signaling in cells [Yamaguchi, 2000].

*Correspondence to: Masayoshi Yamaguchi, Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan.

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The regucalcin gene is localized on the proximal end of rat chromosome Xq 11.1-12 [Shimokawa et al., 1995]. Promotor activity of the regucalcin gene has been shown to be stimulated by the Ca^{2+} signal [Murata and Yamaguchi, 1999]. The regucalcin mRNA is mainly expressed in the liver and kidney of rats [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. It has been shown that regucalcin mRNA is expressed in the kidney cortex but not the medulla of rats, and the expression is stimulated by the administration of calcium chloride to rats [Yamaguchi and Kurota, 1995].

There is growing evidence that regucalcin plays an important role in the regulation of liver cell functions [Yamaguchi, 1992; Yamaguchi, 2000]. The role of regucalcin in kidney cells, however, has not been fully clarified. Kidney cortex constitutes nephron in renal cortex cells is stimulated by calcium administration to rats [Yamaguchi and Kurota, 1995]. Re-

cently, it has been shown that regucalcin can stimulate that ATP-dependent Ca^{2+} pump in the basolateral membranes and microsomes of rat kidney cortex [Kurota and Yamaguchi, 1997a, 1997b]. Presumably, regucalcin has a role in the regulation of cellular Ca^{2+} homeostasis in rat kidney cortex.

An ATP-dependent Ca^{2+} uptake system is present in the mitochondria of rat kidney cortex [Nicholls and Akerman, 1982; van Os, 1987]. Whether this system is regulated by regucalcin is unknown. The present study was undertaken to clarify the effect of regucalcin on ATP-dependent Ca^{2+} uptake activity in the mitochondria of rat kidney cortex. We found that regucalcin had a stimulatory effect on the mitochondrial Ca^{2+} uptake activity in rat kidney cortex.

MATERIALS AND METHODS

Chemicals

Adenosine-5′-triphosphate (ATP), ruthenium red, lanthanum chloride ($LaCl_3$), dithiothreitol, digitonin, dibutyryl cyclic adenosine-5′-monophosphate (DcAMP), 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 a 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; Yamaguchi and Isogai, 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 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, 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].

Preparation of Mitochondria

Rats were killed by cardiac puncture, and the kidneys were removed immediately and decapsulated. The kidney cortices were 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 10 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 800*g* for 10 min to remove nuclei, unbroken cells, and cell debris. The resultant supernatant was centrifuged at 8,500*g* for 10 min to separate the mitochondrial fraction. The mitochondrial fraction was resuspended in 5 mM $MgCl_2$, 50 mM KCl, and 10 mM HEPES, pH 7.0.

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 (800–900 μg as protein) in the absence or presence of regucalcin (50–250 nM) [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+} + 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. 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: 800–900 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). ATP (2 mM), adjusted to pH 7.4 with KOH, was added to initiate energy-dependent Ca^{2+} uptake. After 10 min, a 0.5-ml sample was filtered through a 0.22 μm prewetted Milipore 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 mitochondria.

Statistical Analysis

Data were expressed as the mean ± S.E.M. Statistical differences were analyzed using Student's *t*-test. A *P* value of 0.05 was considered statistically significant.

RESULTS

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

The effect of $CaCl_2$ on Ca^{2+}-ATPase activity in the mitochondria of rat kidney cortex is shown in Fig. 1. The addition of $CaCl_2$ (5, 10, 25, and 50 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 (50, 100, and 250 μM) produced a significant elevation of Ca^{2+}-ATPase activity (Fig. 2A). Meanwhile, Mg^{2+}-ATPase activity in renal cortex mitochondria was not appreciably altered by the addition of regucalcin (50–250 nM) in the presence of 1 mM EGTA (Fig. 2B).

Ruthenium red and lanthanum chloride ($LaCl_3$) are inhibitors of the mitochondria Ca^{2+} uniporter [Nicholls and Akerman, 1982]. Ca^{2+}-ATPase activity in renal cortex mitochondria was significantly decreased by the addition of ruthenium red (10^{-7}–10^{-5} M; Fig. 3A) or $LaCl_3$ (10^{-6} and 10^{-5} M) (Fig. 4A). The effect of regucalcin (100 nM) in increasing Ca^{2+}-ATPase activity was not entirely seen in the presence of ruthenium red (10^{-6} M) or $LaCl_3$ (10^{-6} M) (Fig. 3B and 4B), indicating that regucalcin acts on ruthenium red- or $LaCl_3$-sensitive Ca^{2+}-ATPase (uniporter) in the mitochondria of renal cortex.

The effect of regucalcin on ATP-dependent $^{45}Ca^{2+}$ uptake by renal cortex mitochondria is shown in Figure 5. The addition of ATP to the reaction mixture containing $^{45}Ca^{2+}$ led to mitochondrial $^{45}Ca^{2+}$ uptake (Fig. 5A). This up-

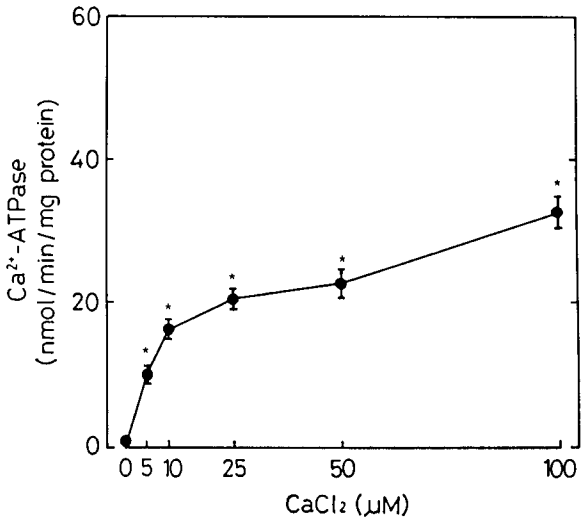

Fig. 1. Alteration in Ca^{2+}-ATPase activity with increasing concentration of Ca^{2+} addition in the mitochondria of rat renal cortex. $CaCl_2$ was added to the enzyme reaction mixture yielding concentrations of 5, 10, 25, 50, and 100 μM. Each value is the mean ± SEM of five experiments with separate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

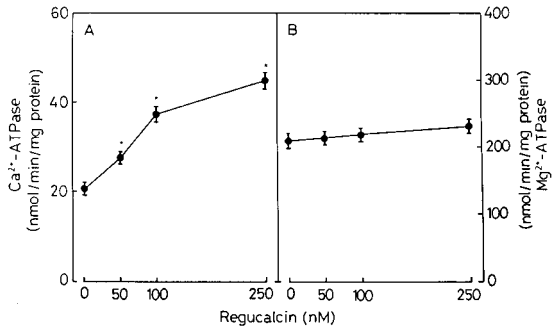

Fig. 2. Effect of regucalcin on Ca^{2+}-ATPase and Mg-ATPase activities in the mitochondria of rat renal cortex. Regucalcin was added to the enzyme reaction mixture yielding concentrations of 50, 100, and 250 μM in the presence of 50 μM $CaCl_2$. Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, as compared with the control value without regucalcin addition.

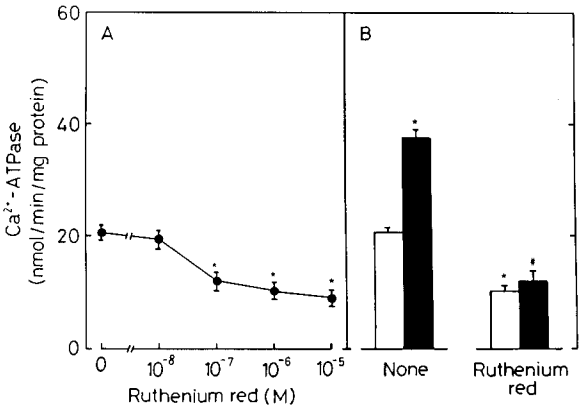

Fig. 3. Effect of ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex. **A:** Ruthenium red was added to the enzyme reaction mixture yielding concentrations of 10^{-8}–10^{-5} M in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (100 nM) 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.

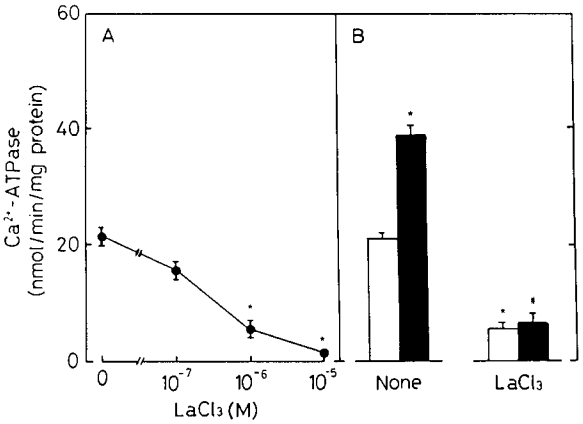

Fig. 4. Effect of lanthanum chloride ($LaCl_3$) on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex. **A:** $LaCl_3$ was added to the enzyme reaction mixture yielding concentrations of 10^{-7}–10^{-5} M in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (100 nM) in the absence or presence of $LaCl_3$ (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.

take was significantly increased by the presence of regucalcin (50, 100, and 250 μM; Fig. 5A). The effect of regucalcin (100 μM) in elevating mitochondrial $^{45}Ca^{2+}$ uptake was completely prevented by the presence of ruthenium

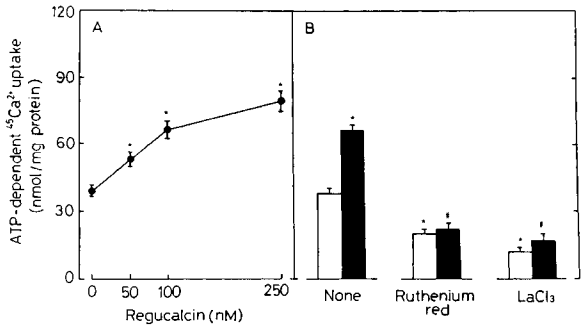

Fig. 5. Effect of regucalcin on the ATPase-dependent $^{45}Ca^{2+}$ uptake in the mitochondria of rat renal cortex. $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods. **A:** The mitochondria were incubated for 10 min after the addition of ATP in the absence or presence of regucalcin (50, 100, and 250 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^{-6} M) in the absence or presence of regucalcin (100 nM). 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.

red (10^{-6} M). $LaCl_3$ (10^{-6} M) alone had a significant inhibitory effect on mitochondrial $^{45}Ca^{2+}$ uptake (Fig. 5B). Thus, regucalcin had a stimulatory effect on Ca^{2+} uptake activity in the mitochondria of rat renal cortex.

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

The effect of digitonin on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex is shown in Figure 6. Digitonin has a solubilizing 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}%), the effect of regucalcin in elevating Ca^{2+}-ATPase activity was not seen.

The effect of dithiothreitol (DTT), a protecting reagent of sulfhydryl (SH) groups, on the regucalcin-raised Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex is shown in Figure 7. The presence of DTT (2.5 and 5.0 mM) in the enzyme reaction mixture caused a significant increase in Ca^{2+}-ATPase activity (Fig. 7A). In the presence of DTT (5.0 mM), the effect of regucalcin on Ca^{2+}-ATPase activity was not further enhanced (Fig. 7B).

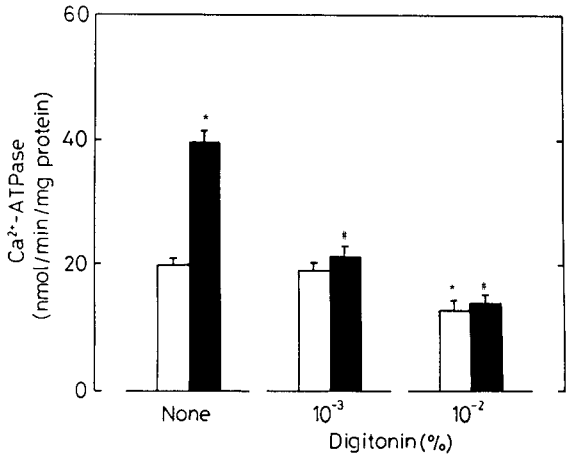

Fig. 6. Effect of digitonin on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex. The enzyme reaction mixture contained either vehicle or digitonin (10^{-3}% or 10^{-2}%) in the absence or presence of regucalcin (100 nM) with 50 μM $CaCl_2$. Each value is the mean ± 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.

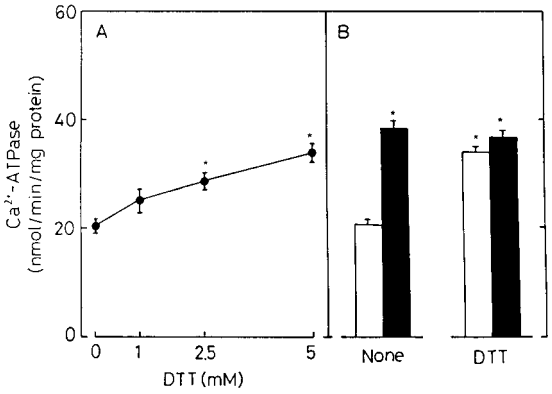

Fig. 7. Effect of dithiothreitol (DTT) on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex. **A:** DTT was added to the enzyme reaction mixture yielding concentrations of 1, 2.5, and 5.0 mM in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (100 nM) in the absence or presence of DTT (5.0 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.

The effect of vanadate, an inhibitor of Ca^{2+}-dependent phosphorylation of (Ca^{2+} + Mg^{2+})-ATPase in plasma membranes [Chen and Junger, 1983], on the regucalcin-raised Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex is shown in Figure 8. The presence of

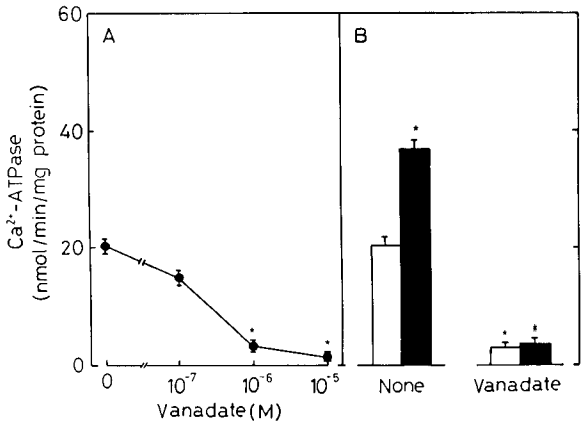

Fig. 8. Effect of vanadate on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex. **A:** Vanadate was added to the enzyme reaction mixture yielding concentrations of 10^{-7}–10^{-5} M in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (100 nM) in the absence or presence of vanadate (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.

vanadate (10^{-6} and 10^{-5} M) led to a significant decrease in Ca^{2+}-ATPase activity (Fig. 8A). The effect of regucalcin (100 nM) in elevating Ca^{2+}-ATPase activity was not seen in the presence of vanadate (10^{-6} M; Fig. 8B).

Effect of Calmodulin or DcAMP on Ca^{2+}-ATPase Activity

The effect of calmodulin, which can modulate Ca^{2+} action [Cheung, 1980], on the regucalcin-elevated Ca^{2+}-ATPase activity in the mitochondria of renal cortex is shown in Figure 9. Ca^{2+}-ATPase activity was significantly raised by the presence of calmodulin (2.5 and 5.0 μg/ml; 0.15 and 0.30 μM) in the enzyme reaction mixture (Fig. 9A). The effect of regucalcin in elevating Ca^{2+}-ATPase activity was not significantly enhanced by the presence of calmodulin (5.0 μg/ml; Fig. 9B).

The effect of trifluoperazine (TFP), an antagonist of calmodulin [Vincenzi, 1982], on the regucalcin-increased Ca^{2+}-ATPase in the mitochondria of renal cortex is shown in Figure 10. The presence of TFP (50 or 100 μM) in the enzyme reaction mixture caused a significant decrease in Ca^{2+}-ATPase activity (Fig. 10A). In the presence of TFP (50 μM), regucalcin (100 nM) had a significant effect on mitochon-

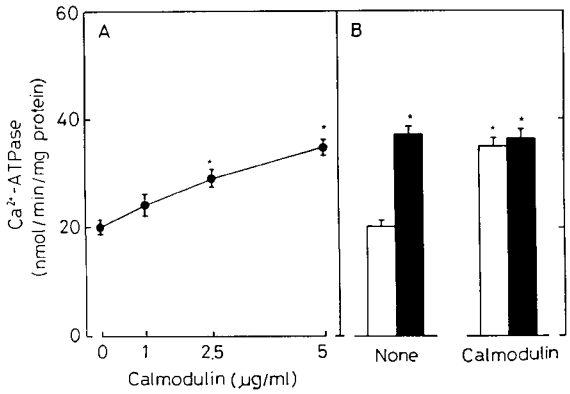

Fig. 9. Effect of calmodulin, a Ca^{2+}-binding protein, on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex. **A:** Calmodulin was added to the enzyme reaction mixture yielding concentrations of 1, 2.5, and 5.0 μg/ml in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (100 nM) in the absence or presence of calmodulin (5.0 μg/ml). 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.

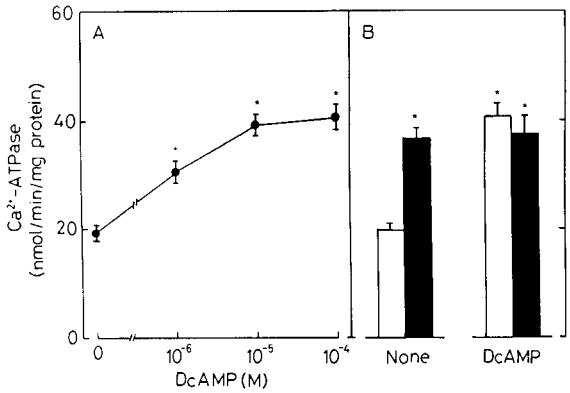

Fig. 11. Effect of dibutyryl cyclic AMP (DcAMP) on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex. **A:** DcAMP was added to the enzyme reaction mixture yielding concentrations of 10^{-6}–10^{-4} M in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (100 nM) in the absence or presence of DcAMP (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.

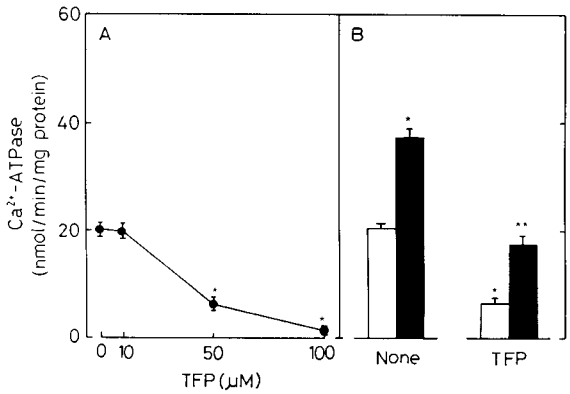

Fig. 10. Effect of trifluoperazine (TFP), an antagonist of calmodulin, on the regucalcin-increased Ca^{2+}-ATPase activity in the mitocondria of rat renal cortex. **A:** TFP was added to the enzyme reaction mixture yielding concentrations of 10, 50, and 100 μM in the presence of 50 μM $CaCl_2$. **B:** The enzyme reaction mixture contained either vehicle or regucalcin (100 nM) in the absence or presence of TFP (50 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 TFP alone.

drial Ca^{2+}-ATPase activity (Fig. 10B). Thus, the effect of regucalcin in elevating mitochondrial Ca^{2+}-ATPase activity was not involved in calmodulin that might be present in mitochondria.

The effect of DcAMP on the regucalcin-increased Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex is shown in Figure 11. The presence of DcAMP (10^{-6}–10^{-4} M) in the enzyme reaction mixture led to a significant increase in Ca^{2+}-ATPase activity (Fig. 11A). In the presence of DcAMP (10^{-4} M), mitochondrial Ca^{2+}-ATPase activity was not significantly raised by the addition of regucalcin (100 nM; Fig. 11B).

DISCUSSION

The kidney cortex possesses nephrons including glomeruli and tubules. The reabsorption of urinary calcium in kidney is promoted by transcellular Ca^{2+} transport in the epithelial cells of renal tubules [Ng et al., 1982; Agus et al., 1997]. The regulation of intracellular Ca^{2+} homeostasis is important in the promotion of transcellular Ca^{2+} transport. 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 transporting it across the plasma membrane or accumulate it inside intracellular organelles such as the mitochondria and endoplasmic reticulum [Carafoli and Zurini, 1982; van Os, 1987].

ATP-dependent Ca^{2+} uptake by mitochondrial and nonmitochondrial stores is demonstrated by using saponin-permeabilized rat cortical kidney cells [van Os, 1987]. Regucalcin

is expressed in the kidney cortex including nephrons of rats [Yamaguchi and Kurota, 1995], and the protein may play a physiologic role in the regulation of intracellular Ca^{2+} homeostasis in the epithelial cells of kidney tubules by activating ATP-dependent Ca^{2+} transport systems in the basolateral membranes and the microsomes. The effect of regucalcin on ATP-dependent Ca^{2+} uptake activity in renal cortex mitochondria, however, is not clarified.

The present study clearly demonstrates that regucalcin can stimulate ATP-dependent Ca^{2+} uptake in the mitochondria of rat kidney cortex. 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 protein gradient) that is responsible for driving Ca^{2+} electrophoretically [Brand and Lehninger, 1975; Nicholls, 1978]. The effect of regucalcin in elevating mitochondrial Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake was completely blocked by ruthenium red or $LaCl_3$, which are specific inhibitors of the Ca^{2+} uniporter in the mitochondria [Nicholls and Akerman, 1982]. This finding suggests that regucalcin stimulates Ca^{2+}-ATPase-related Ca^{2+} uniporter activity in renal cortex mitochondria.

A possible mechanism of regucalcin action in increasing mitochondrial Ca^{2+}-ATPase activity was examined. The activating effect of regucalcin on mitochondrial Ca^{2+}-ATPase was not entirely seen in the presence of digitonin, a solubilization reagent of membranous lipids [Murphy et al., 1980], in the enzyme reaction mixture. This result suggests that regucalcin increased Ca^{2+}-ATPase activity by its binding to mitochondrial membranous lipids. In addition, the effect of regucalcin on Ca^{2+}-ATPase activity was not significantly enhanced by the presence of DTT (a protecting reagent of SH groups, which can increase the enzyme activity) suggesting that regucalcin acts on the SH groups of Ca^{2+}-ATPase. Moreover, the effect of regucalcin was completely blocked by the presence of vanadate, an inhibitor of Ca^{2+}-dependent phosphorylation of $(Ca^{2+} + Mg^{2+})$-ATPase [Chen and Junger, 1983], supporting the view that regucalcin stimulates Ca^{2+}-dependent phosphorylation of Ca^{2+}-ATPase in renal cortex mitochondria. Presumably regucalcin binds to the membranous lipids of renal cortex mitochondria, and it acts on the SH groups, which are active sites of Ca^{2+}-ATPase. This leads to the stimulation of Ca^{2+}-dependent phosphorylation of the enzyme.

Calmodulin or DcAMP could increase Ca^{2+}-ATPase activity in the mitochondria of rat renal cortex, suggesting an involvement of intracellular signaling factors in the regulation of the mitochondrial ATP-dependent Ca^{2+}-uptake system. The activatory effect of regucalcin on mitochondrial Ca^{2+}-ATPase activity was not significantly enhanced by calmodulin or DcAMP. Regucalcin, calmodulin, and cyclic AMP may regulate reciprocally Ca^{2+} uptake activity in the mitochondria of renal cortex cells. The effect of regucalcin in elevating mitochondrial Ca^{2+}-ATPase activity was also seen in the presence of TFP, an antagonist of calmodulin [Vincenzi, 1982], which can decrease the enzyme activity. This result indicates that the effect of regucalcin is independent on calmodulin in the mitochondria of renal cortex cells. Mitochondrial Ca^{2+} sequestration may be individually activated by regucalcin or calmodulin, which is a Ca^{2+}-binding protein. Both proteins may be important as activators in mitochondrial ATP-dependent Ca^{2+} sequestration.

The reabsorption of urinary calcium in kidney is promoted by transcellular Ca^{2+} transport in the epithelial cells of renal tubules [Ng et al., 1982; Agus et al., 1997]. Regucalcin can stimulate Ca^{2+} transport (efflux) across the basolateral membranes of rat renal cortex [Kurota and Yamaguchi, 1997a] and the mitochondrial ATP-dependent Ca^{2+} sequestration [Kurota and Yamaguchi, 1997b]. Moreover, regucalcin had a stimulatory effect on mitochondrial ATP-dependent Ca^{2+} uptake in rat renal cortex. Thus, regucalcin may play a physiologic role in the regulation of intracellular Ca^{2+} homeostasis in the epithelial cells of kidney tubules by activating ATP-dependent Ca^{2+}-transport systems in the basolateral membranes, microsomes, and mitochondria.

In conclusion, it has been demonstrated that regucalcin can increase Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake in the mitochondria of rat renal cortex cells.

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