

# Regucalcin increases $\text{Ca}^{2+}$ -ATPase activity in the heart mitochondria of normal and regucalcin transgenic rats

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**Abstract.** The role of regucalcin, a regulatory protein in intracellular signaling system, in the regulation of  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria was investigated. Mitochondrial  $\text{Ca}^{2+}$ -ATPase activity was significantly increased by increasing concentrations of  $\text{CaCl}_2$  (2.5-50  $\mu\text{M}$ ). An increase in the enzyme activity was saturated at 50  $\mu\text{M}$   $\text{CaCl}_2$ . The addition of regucalcin ( $10^{-11}$ - $10^{-8}$  M) in the enzyme reaction mixture caused a significant increase in  $\text{Ca}^{2+}$ -ATPase activity in heart mitochondria in the presence of 50  $\mu\text{M}$   $\text{CaCl}_2$ . Regucalcin did not have a significant effect on mitochondrial  $\text{Mg}^{2+}$ -ATPase activity. Regucalcin (10<sup>-9</sup> M) did not have a significant effect on  $\text{Ca}^{2+}$ -ATPase activity in the presence of digitonin (10<sup>-3</sup> or 10<sup>-2</sup> %), which is a solubilization effect on membranous lipids. The effect of regucalcin in increasing mitochondrial  $\text{Ca}^{2+}$ -ATPase activity was not observed in the presence of ruthenium red (10<sup>-7</sup> M) or lanthanum chloride (10<sup>-7</sup> M), which is an inhibitor of  $\text{Ca}^{2+}$  uniporter. The effect of regucalcin (10<sup>-9</sup> M) in increasing mitochondrial  $\text{Ca}^{2+}$ -ATPase activity was not significantly enhanced in the presence of calmodulin (5  $\mu\text{g}/\text{ml}$ ) or dibutyryl cyclic AMP (10<sup>-4</sup> M), which is an intracellular signaling factor that can cause a significant increase in the enzyme activity. Mitochondrial regucalcin localization was significantly increased in the heart of regucalcin transgenic rats as compared with that of normal rats using Western blot analysis.  $\text{Ca}^{2+}$ -ATPase activity was significantly increased in the heart mitochondria of regucalcin transgenic rats. This study demonstrates that regucalcin has an activating effect on  $\text{Ca}^{2+}$ -ATPase in rat heart mitochondria, suggesting its role in the regulation of heart mitochondrial function.

## Introduction

Regucalcin was discovered as a novel  $\text{Ca}^{2+}$ -binding protein not including the EF-hand motif, which differs from calmodulin, in the liver cytosol of rats (1). The name regucalcin was proposed for this  $\text{Ca}^{2+}$ -binding protein, which can regulate  $\text{Ca}^{2+}$  and/or calmodulin effects on various enzymes in liver cells (2,3). In recent years, regucalcin has been demonstrated to play a multifunctional role as a regulatory protein in intracellular signaling pathway in many cell types (4-6). The gene of regucalcin is highly conserved in vertebrate species (7,8). Rat and human regucalcin genes are localized on chromosome X (9,10). The organization of the rat regucalcin gene consists of seven exons and six introns, and several consensus regulatory elements exist in the upstream of the 5'-flanking region of the gene (11). AP-1 (12), NF1-A1 (13) and RGPR-p117 (14) have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity.

Regucalcin is greatly expressed in the liver and only to a small extent in the kidney cortex (15,16). In addition, a lower expression of regucalcin is found in rat brain (17), heart muscle (18) and bone tissue (19). Regucalcin plays a role in the maintenance of intracellular  $\text{Ca}^{2+}$  homeostasis, the inhibitory regulation of various protein kinases (including  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase, protein kinase C and protein tyrosine kinase), protein phosphatases, nitric oxide synthase, protein synthesis, and control of the enhancement of nuclear DNA and RNA synthesis in proliferative cells (3-6,20-23). Moreover, regucalcin has been shown to have a suppressive effect on proliferation and apoptosis in normal and transformed cells (24,25). Regucalcin may play a pivotal role in the regulation of cell function.

The role of regucalcin in heart muscle function has not been fully clarified, although regucalcin mRNA and its protein are expressed in rat heart muscle (18). Recently, it has been shown that regucalcin can activate  $\text{Ca}^{2+}$ -ATPase, which plays a role as a  $\text{Ca}^{2+}$  pump, in rat heart microsomes (18), and that it increases superoxide dismutase activity in rat heart cytosol (26). Moreover, regucalcin has been shown to have inhibitory effects on protein phosphatase (27) and nitric oxide synthase (28) activities in rat heart cytosol. Regucalcin may have a regulatory effect on heart muscle function.

This study was undertaken to determine whether regucalcin can regulate the activity of  $\text{Ca}^{2+}$ -ATPase, which is related to

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the transportation of  $\text{Ca}^{2+}$  in heart mitochondria. We found that regucalcin has an activating effect on  $\text{Ca}^{2+}$ -ATPase in the heart mitochondria of normal (wild-type) and regucalcin transgenic rats.

## Materials and methods

**Chemicals.** Adenosin triphosphate magnesium salt (Mg-ATP), ruthenium red, lanthanum chloride ( $\text{LaCl}_3$ ), digitonin, calmodulin (50,000 units/mg protein from bovine brain), and dibutyryl cyclic adenosine monophosphate (DcAMP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Calcium chloride and other chemicals were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Reagents used were dissolved in distilled water and then passed through an ion-exchange resin to remove metal ions.

**Animals.** Wistar strain male rats (4 weeks old), purchased from Japan SLC Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid, Oriental Yeast Co., Tokyo, Japan) containing 57.5% carbohydrate, 1.1% calcium and 1.1% phosphorus and distilled water, freely. Animals were used for experiments at 5 weeks old.

**Isolation of regucalcin.** Regucalcin is markedly expressed in rat liver cytosol (1). Regucalcin was isolated from rat liver cytosol. Livers were perfused with Tris-HCl buffer (pH 7.4), containing 100 mM Tris, 120 M NaCl, 4 mM KCl, cooled to 4°C. Livers were removed, cut into small pieces, suspended (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  $\times g$  in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000  $\times g$  for 60 min. The resulting supernatant was heated at 60°C for 10 min and recentrifuged at 38,000  $\times g$  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 (1).

**Preparation of heart mitochondria.** Rats were sacrificed by cardiac puncture, and the heart 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-piperazin-ethanesulfonic 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 (29). The homogenate was centrifuged at 800  $\times g$  for 10 min to remove nuclei, unbroken cells, and cell debris. The resultant supernatant was centrifuged at 8,500  $\times g$  for 10 min to sediment the mitochondrial fraction. The mitochondrial fraction was resuspended in 5 mM  $\text{MgCl}_2$ , 50 mM KCl, and 10 mM HEPES, pH 7.0, to a final protein concentration of 2.0-2.5 mg/ml. The 8,500  $\times g$  supernatant was spun at 105,000  $\times g$  for 60 min, and the supernatant fraction (cytosol) was pooled to assay regucalcin level. Protein concentration was determined using the method of Lowry *et al* (30).

**Assay of  $\text{Ca}^{2+}$ -ATPase activity.**  $\text{Mg}^{2+}$ -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  $\text{MgCl}_2$ , 6 mM succinate, 8 mM Mg-ATP, and the mitochondria (200-250  $\mu\text{g}$  as protein) in the presence or absence of regucalcin ( $10^{-11}$ - $10^{-8}$  M). ( $\text{Ca}^{2+}$ + $\text{Mg}^{2+}$ )-ATPase activity was measured in the same medium with addition of 50  $\mu\text{M}$   $\text{CaCl}_2$  in the presence or absence of regucalcin ( $10^{-11}$ - $10^{-8}$  M).  $\text{Ca}^{2+}$ -ATPase activity was calculated as the difference between ( $\text{Ca}^{2+}$ + $\text{Mg}^{2+}$ )-ATPase and  $\text{Mg}^{2+}$ -ATPase (31). Inorganic phosphate was measured using the method of Nakamura and Mori (32). Enzyme activity was expressed as nmol of inorganic phosphate released per minute (min) per milligram (mg) protein of mitochondria.

**Regucalcin transgenic (TG) rats.** Normal rats (Sprague-Dawley, SD) and regucalcin TG rats (SD) were supplied from Japan SLC (Hamamatsu, Japan). Regucalcin TG rats were generated as described (33). A 3.6 liner DNA fragment containing the regucalcin (RC)/pCXN2 was used for pronuclear microinjection of rat (SD) embryos to generate transgenic (TG) rats. The embryos were implanted into pseudopregnant female rats. The founder rats were mated with SD rats to produce F1 litters. To identify founder rats and determine transgene copy number, genomic DNA was isolated from tails and amplified by PCR using primer sets that recognized two different regions of the regucalcin cDNA (7). Primers huRC-1 (5'-GGAGGCTATGTTGCCACCATT GGA-3') and huRC-2 (5'-CCCTCCAAGCAGCATGA AGTTG-3') amplified a fragment containing the regucalcin cDNA that was present in the transgene sequence, but absent in the wild type. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Two TG founder rats, one male and one female, carrying the regucalcin fusion gene were obtained. Both founders were fertile, transmitted the transgene at the expected frequency, and bred to homozygote (33). In the experiments, normal rats and regucalcin TG rats of 5 weeks old were used. These rats were sacrificed by cardiac puncture, and the heart was removed immediately. The mitochondria were prepared from heart homogenate to assay  $\text{Ca}^{2+}$ -ATPase activity and regucalcin level.

**Western blot analysis.** Aliquots of heart mitochondria and cytosolic extracts (50  $\mu\text{g}$  of protein) from normal rats or heart mitochondria (20  $\mu\text{g}$  of protein) from regucalcin transgenic rats were mixed with 5X Laemmli sample buffer, boiled for 5 min, and resolved by 12% SDS-PAGE (34). The proteins were then transferred onto a polyvinylidene difluoride membrane at 100 mA for 4 h (35). The membranes were incubated with a polyclonal rabbit anti-regucalcin antibody (11), which was diluted 1:2,000 in 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 0.1% (wt./vol.) Tween-20 (washing buffer), and 5% (wt./vol.) skim milk for 1 h. The membranes were incubated and washed four times with washing buffer. Then, the membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit IgG, which was diluted 1:5,000 in washing buffer containing 5% (wt./vol.) skim milk, and again they were washed. Detection of the protein bands was performed using the enhanced

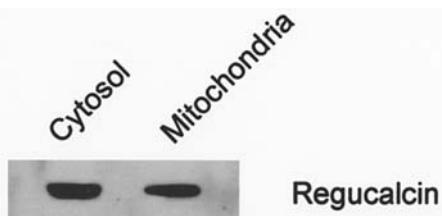


Figure 1. Localization of regucalcin in the mitochondria of rat heart. Proteins ( $50 \mu\text{g}$ ) of the mitochondria or cytosol prepared from rat heart homogenate were analyzed by Western blotting. One of four experiments with separate samples.

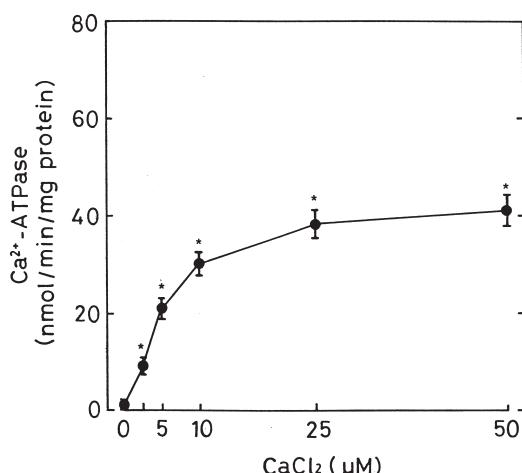


Figure 2. Alteration in  $\text{Ca}^{2+}$ -ATPase activity with increasing concentrations of  $\text{Ca}^{2+}$  addition in rat heart mitochondria.  $\text{CaCl}_2$  was added to the enzyme reaction mixture, yielding concentrations of 2.5, 5, 10, 25, or  $50 \mu\text{M}$ . Each value is the mean  $\pm$  SEM of five experiments with separate rats. \* $P<0.01$  compared with the control (none) value without  $\text{Ca}^{2+}$  addition.

chemiluminescent kit following the manufacturer's instructions. The membranes were exposed to film for 5 min. The size of the detecting protein was determined by running the standard protein molecular of known sizes in parallel.

**Statistical analysis.** Data were expressed as the mean  $\pm$  SEM. Statistical differences were analyzed using Student's t-test. A P-value of  $<0.05$  was considered to indicate statistically significant difference. Also, we used a multiway ANOVA multiple comparison test to compare the treatment groups.

## Results

**Localization of regucalcin in rat heart mitochondria.** Whether regucalcin is localized in the mitochondria of normal rat heart was examined using Western blot analysis (Fig. 1). Regucalcin was detected in the cytosol and mitochondria of rat heart. This result indicates that regucalcin in the cytoplasm is translocated into the mitochondria, suggesting that regucalcin has a role in the regulation of mitochondria function.

**Effect of regucalcin addition on  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria.** The effect of  $\text{Ca}^{2+}$  addition on  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria was examined (Fig. 2).  $\text{Ca}^{2+}$ -ATPase activity in the mitochondria was significantly increased by  $\text{CaCl}_2$  addition. The increase in

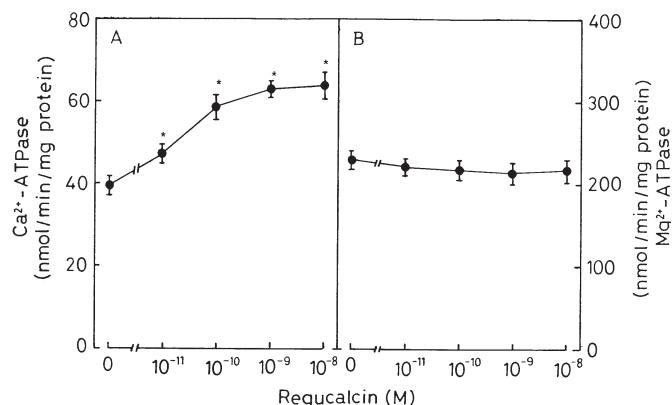


Figure 3. Effect of regucalcin addition on  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria. Regucalcin was added to the enzyme reaction mixture, yielding concentrations of  $10^{-11}$ - $10^{-8}$  M in the presence (A) or absence (B) of  $50 \mu\text{M}$   $\text{CaCl}_2$ . Each value is the mean  $\pm$  SEM of five experiments with separate rats. \* $P<0.01$  compared with the control (none) value without regucalcin addition.

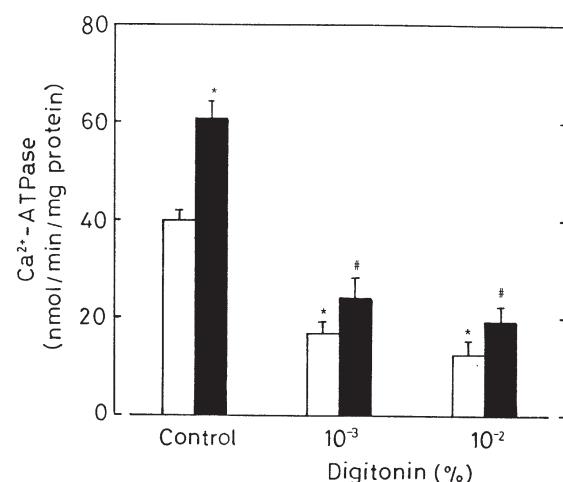


Figure 4. Effect of digitonin on the regucalcin-increased  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria. The enzyme reaction mixture contained either vehicle or digitonin ( $10^{-3}$  or  $10^{-2}$  %) in the presence or absence of regucalcin ( $10^{-9}$  M) with  $50 \mu\text{M}$   $\text{CaCl}_2$  addition. Each value is the mean  $\pm$  SEM of five experiments with separate rats. \* $P<0.01$  compared with the control (none) value. # $P<0.01$  compared with the value obtained from addition of regucalcin alone. White bars, without regucalcin; black bars, with regucalcin.

$\text{Ca}^{2+}$ -ATPase was saturated at the concentrations of 25 and  $50 \mu\text{M}$   $\text{CaCl}_2$ . Regucalcin was added to the enzyme reaction mixture in the presence of  $50 \mu\text{M}$   $\text{CaCl}_2$  (Fig. 3). Mitochondrial  $\text{Ca}^{2+}$ -ATPase activity was significantly increased in the presence of regucalcin ( $10^{-11}$ - $10^{-8}$  M) with  $50 \mu\text{M}$   $\text{CaCl}_2$  addition (Fig. 3A). Meanwhile,  $\text{Mg}^{2+}$ -ATPase activity in the mitochondria was not significantly changed by the addition of regucalcin ( $10^{-11}$ - $10^{-8}$  M) without  $\text{Ca}^{2+}$  addition (Fig. 3B).

**Characterization of regucalcin action on  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria.** The effect of digitonin on the regucalcin-increased  $\text{Ca}^{2+}$ -ATPase activity in heart mitochondria is shown in Fig. 4. Digitonin has a solubilization effect on membranous lipids (36). The presence of digitonin ( $10^{-3}$  or  $10^{-2}$ %) in the enzyme reaction mixture caused a

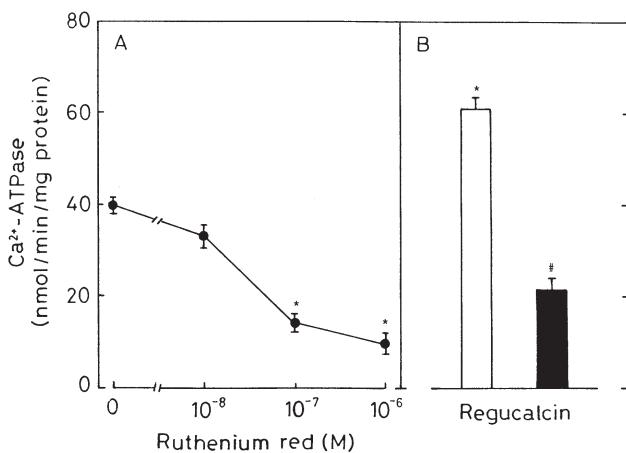


Figure 5. Effect of ruthenium red on the regucalcin-increased  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria. (A) The enzyme reaction mixture contained either vehicle or ruthenium red ( $10^{-8}$ - $10^{-6}$  M) in the presence of  $50 \mu\text{M}$   $\text{CaCl}_2$ . (B) The enzyme reaction mixture contained either regucalcin ( $10^{-9}$  M) or regucalcin ( $10^{-9}$  M) plus ruthenium red ( $10^{-7}$  M) in the presence of  $50 \mu\text{M}$   $\text{CaCl}_2$ . Each value is the mean  $\pm$  SEM of five experiments with separate rats. \* $P<0.01$  compared with the control (none) value. # $P<0.01$  compared with the value obtained from addition of regucalcin alone. White bar, without ruthenium red; black bar, with ruthenium red.

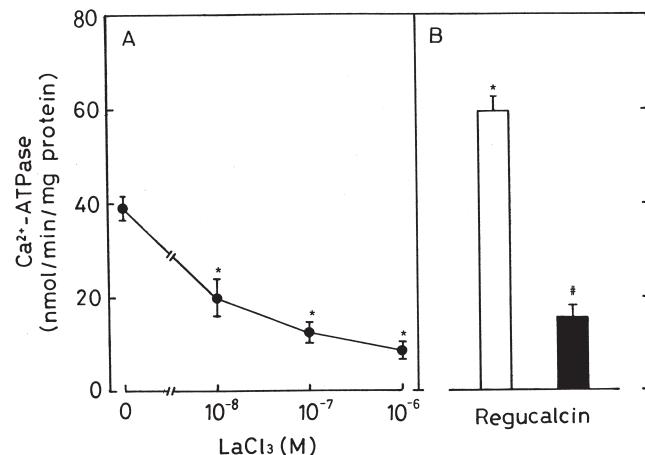


Figure 6. Effect of lanthanum chloride ( $\text{LaCl}_3$ ) on the regucalcin-increased  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria. (A) The enzyme reaction mixture contained either vehicle or  $\text{LaCl}_3$  ( $10^{-8}$ - $10^{-6}$  M) in the presence of  $50 \mu\text{M}$   $\text{CaCl}_2$ . (B) The enzyme reaction mixture contained either regucalcin ( $10^{-9}$  M) or regucalcin ( $10^{-9}$  M) plus  $\text{LaCl}_3$  ( $10^{-7}$  M) in the presence of  $50 \mu\text{M}$   $\text{CaCl}_2$ . Each value is the mean  $\pm$  SEM of five experiments with separate rats. \* $P<0.01$  compared with the control (none) value. # $P<0.01$  compared with the value obtained from addition of regucalcin alone. White bar, without  $\text{LaCl}_3$ ; black bar, with  $\text{LaCl}_3$ .

significant decrease in mitochondrial  $\text{Ca}^{2+}$ -ATPase activity. In the presence of digitonin ( $10^{-3}$  or  $10^{-2}$  M), regucalcin could not increase  $\text{Ca}^{2+}$ -ATPase activity.

Ruthenium red or lanthanum chloride ( $\text{LaCl}_3$ ) is an inhibitor of the mitochondrial  $\text{Ca}^{2+}$  uniporter (37). Heart mitochondrial  $\text{Ca}^{2+}$ -ATPase activity was markedly decreased in the presence of ruthenium red ( $10^{-7}$  or  $10^{-6}$  M) (Fig. 5A) or  $\text{LaCl}_3$  ( $10^{-7}$  or  $10^{-6}$  M) (Fig. 6A). The effect of regucalcin ( $10^{-9}$  M) in increasing  $\text{Ca}^{2+}$ -ATPase activity was not markedly inhibited in the presence of

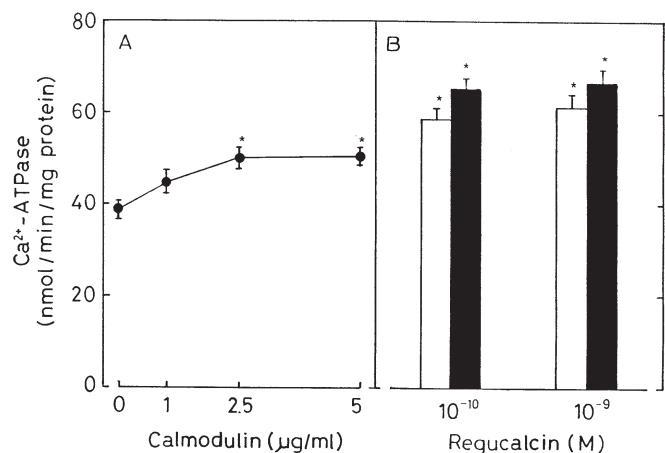


Figure 7. Effect of calmodulin addition on the regucalcin-increased  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria. (A) The enzyme reaction mixture contained either vehicle or calmodulin (1, 2.5, or 5  $\mu\text{g}/\text{ml}$ ) in the presence of  $50 \mu\text{M}$   $\text{CaCl}_2$ . (B) The enzyme reaction mixture contained either vehicle or calmodulin (5  $\mu\text{g}/\text{ml}$ ) in the presence of regucalcin ( $10^{-10}$  or  $10^{-9}$  M) with  $50 \mu\text{M}$   $\text{CaCl}_2$ . Each value is the mean  $\pm$  SEM of five experiments with separate rats. \* $P<0.01$  compared with the control (none) value.

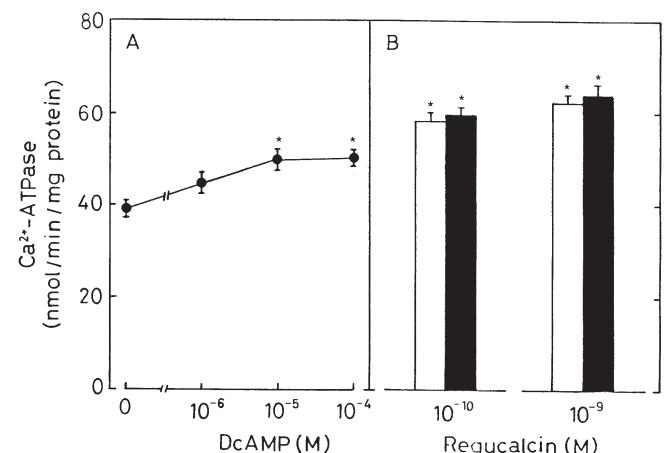


Figure 8. Effect of dibutyryl cyclic AMP (DcAMP) on the regucalcin-increased  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria. (A) The enzyme reaction mixture contained either vehicle or DcAMP ( $10^{-6}$ - $10^{-4}$  M) in the presence of  $50 \mu\text{M}$   $\text{CaCl}_2$ . (B) The enzyme reaction mixture contained either vehicle or DcAMP ( $10^{-4}$  M) in the presence of regucalcin ( $10^{-10}$  or  $10^{-9}$  M) with  $50 \mu\text{M}$   $\text{CaCl}_2$  addition. Each value is the mean  $\pm$  SEM of five experiments with separate rats. \* $P<0.01$  compared with the control (none) value.

ruthenium red ( $10^{-7}$  M) (Fig. 5B) or  $\text{LaCl}_3$  ( $10^{-7}$  M) (Fig. 6B), suggesting that regucalcin acts on ruthenium red- or  $\text{LaCl}_3$ -sensitive  $\text{Ca}^{2+}$ -ATPase (uniporter) in the mitochondria.

**Effect of signaling factors on regucalcin-increased  $\text{Ca}^{2+}$ -ATPase activity in rat heart mitochondria.** The presence of calmodulin (2.5 or 5  $\mu\text{g}/\text{ml}$ ) (Fig. 7A) or DcAMP ( $10^{-5}$  or  $10^{-4}$  M) (Fig. 8A) in the enzyme reaction mixture caused a significant increase in  $\text{Ca}^{2+}$ -ATPase activity in the mitochondria. In the presence of calmodulin (5  $\mu\text{g}/\text{ml}$ ) (Fig. 7B) or DcAMP ( $10^{-4}$  M) (Fig. 8B), the effect of regucalcin ( $10^{-9}$  M) in increasing mitochondrial  $\text{Ca}^{2+}$ -ATPase activity was not significantly enhanced.

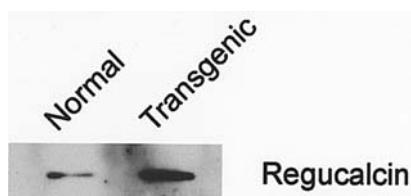


Figure 9. Increase in regucalcin in the heart mitochondria of regucalcin transgenic rats. Proteins (20 µg) of the mitochondria prepared from the hearts of normal or regucalcin transgenic rats were analyzed using Western blot analysis. The figure shows one of four experiments with separate rats. The densitometric data were  $327 \pm 10.1$  (% of normal rats; mean  $\pm$  SEM of four experiments). Data were significant ( $P < 0.01$ ) as compared with that of normal rats.

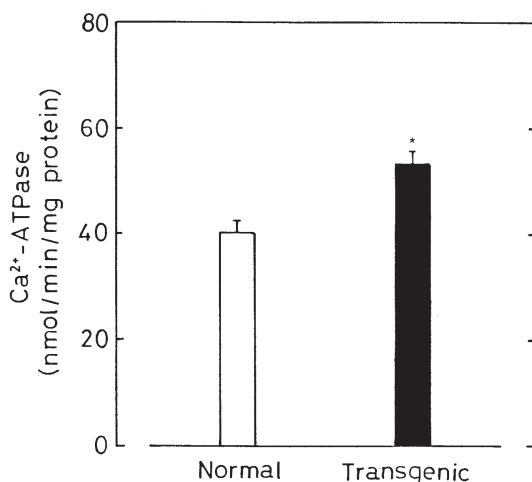


Figure 10. Increase in Ca<sup>2+</sup>-ATPase activity in the heart mitochondria of regucalcin transgenic rats. The enzyme activity was measured in the mixture containing the mitochondria obtained from normal or regucalcin transgenic rats. Each value is the mean  $\pm$  SEM of four rats. \* $P < 0.01$  compared with the value obtained from normal rats.

*Alteration in Ca<sup>2+</sup>-ATPase activity in the heart mitochondria of regucalcin transgenic rats.* The change in regucalcin localization in the heart mitochondria of regucalcin transgenic rats is shown in Fig. 9. Mitochondrial regucalcin localization was significantly increased in regucalcin transgenic rats as compared with that of normal rats. Ca<sup>2+</sup>-ATPase activity in the mitochondria was significantly increased in regucalcin transgenic rats (Fig. 10).

## Discussion

Regucalcin is expressed in the heart of rats (18). The role of regucalcin in the regulation of heart muscle function, however, has not been clarified fully. Recent studies demonstrate that regucalcin has activating effects on microsomal Ca<sup>2+</sup>-ATPase (18) and cytosolic superoxide dismutase (26) in rat heart, and that it has inhibitory effects on cytosolic protein phosphatase (27) and nitric oxide synthetase (28) in the heart. This study, moreover, demonstrates that regucalcin has an activating effect on Ca<sup>2+</sup>-ATPase, which is involved in Ca<sup>2+</sup> transport, in the mitochondria of rat heart. Regucalcin may play a role in the regulation of heart muscle function.

The addition of regucalcin, which is in the range of physiologic levels (16), to the enzyme reaction mixture was found to increase Ca<sup>2+</sup>-ATPase activity in rat heart mitochondria. However, mitochondrial Mg<sup>2+</sup>-ATPase activity was not significantly altered by the addition of regucalcin. The effect of regucalcin in increasing mitochondrial Ca<sup>2+</sup>-ATPase activity was not seen in the presence of digitonin, which is a solubilization effect on membranous lipids (36), in the enzyme reaction mixture. This result suggests that regucalcin binds to mitochondrial membranes and increases Ca<sup>2+</sup>-ATPase activity in rat heart.

Ruthenium red or LaCl<sub>3</sub> is an inhibitor of the mitochondrial Ca<sup>2+</sup> uniporter (37). Heart mitochondrial Ca<sup>2+</sup>-ATPase activity was markedly decreased by the addition of those inhibitors in the enzyme reaction mixture. This result suggests that regucalcin activates Ca<sup>2+</sup>-ATPase, which stimulates Ca<sup>2+</sup> uptake that is mediated through the uniporter in rat heart mitochondria.

Calmodulin (38) and DcAMP (39) are intracellular signaling factors. The presence of calmodulin or DcAMP in the enzyme reaction mixture caused a significant increase in Ca<sup>2+</sup>-ATPase activity in rat heart mitochondria. The effect of regucalcin in increasing mitochondrial Ca<sup>2+</sup>-ATPase activity was not significantly enhanced in the presence of calmodulin or DcAMP. Presumably, regucalcin does not modulate the effect of calmodulin or cyclic AMP in the regulation of Ca<sup>2+</sup>-ATPase activity in rat heart mitochondria. It is speculated that regucalcin has a role in the regulation of heart mitochondrial Ca<sup>2+</sup>-ATPase activity, independent of calmodulin or cyclic AMP, which is a signaling factor in the cells.

Regucalcin was found to localize in the mitochondria of rat heart. This localization was markedly increased in the heart mitochondria of regucalcin transgenic rats. Mitochondrial Ca<sup>2+</sup>-ATPase activity was significantly enhanced in the heart of regucalcin transgenic rats. This finding demonstrates that endogenous regucalcin can activate Ca<sup>2+</sup>-ATPase in the heart mitochondria of rats.

Regucalcin has been shown to activate Ca<sup>2+</sup>-ATPase and Ca<sup>2+</sup> uptake in the heart microsomes of rats (18). In addition, regucalcin was found to increase Ca<sup>2+</sup>-ATPase activity in rat heart mitochondria. Microsomes and mitochondria are important for Ca<sup>2+</sup> storage in many cell types. Regucalcin may play a physiologic role in the regulation of Ca<sup>2+</sup> homeostasis in rat heart muscle cells. The expression of regucalcin is increased through Ca<sup>2+</sup> and other signaling factors in cells (6,40). It is speculated that an increase in intracellular Ca<sup>2+</sup> levels induces expression of regucalcin, and that regucalcin regulates Ca<sup>2+</sup> homeostasis due to activating mitochondrial Ca<sup>2+</sup>-ATPase and microsomal Ca<sup>2+</sup>-ATPase (18) in rat heart cells.

In conclusion, it has been demonstrated that regucalcin increases Ca<sup>2+</sup>-ATPase activity in the heart mitochondria of normal and regucalcin transgenic rats.

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