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# Role of Regucalcin as an Activator of Sarcoplasmic Reticulum $Ca^{2+}$-ATPase Activity in Rat Heart Muscle

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**Abstract** The expression of regucalcin, a regulatory protein of $Ca^{2+}$ signaling, and its effect on $Ca^{2+}$ pump activity in the microsomes (sarcoplasmic reticulum) of rat heart muscle was investigated. The expression of regucalcin mRNA was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) analysis in heart muscle using rat regucalcin-specific primers. Results with Western blot analysis showed that regucalcin protein was present in the cytoplasm, although it was not detected in the microsomes. Microsomal $Ca^{2+}$-ATPase activity was significantly increased in the presence of regucalcin ($10^{-10}$–$10^{-8}$ M) in the enzyme reaction mixture. This increase was not seen in the presence of thapsigargin (TP) ($10^{-5}$ M), a specific inhibitor of the microsomal $Ca^{2+}$ pump enzyme. Regucalcin ($10^{-10}$–$10^{-8}$ M) significantly stimulated ATP-dependent $^{45}Ca^{2+}$ uptake by the microsomes. The effect of regucalcin ($10^{-8}$ M) in increasing microsomal $Ca^{2+}$-ATPase activity was completely prevented in the presence of digitonin ($10^{-3}$ or $10^{-2}$%), which has a solubilizing effect on membranous lipid, or *N*-ethylmaleimide (NEM), a modifying reagent of sulfhydryl (SH) groups. Dithiothreitol (DTT; 5 mM), a protecting reagent of SH groups, increased markedly $Ca^{2+}$-ATPase activity. In the presence of DTT (5 mM), regucalcin could not significantly enhance the enzyme activity. Also, the effect of regucalcin in increasing $Ca^{2+}$-ATPase activity was completely inhibited by the addition of vanadate (1 mM), an inhibitor of phosphorylation of enzyme. In addition, the effect of regucalcin on $Ca^{2+}$-ATPase activity was not significantly modulated in the presence of dibutyryl cyclic AMP ($10^{-4}$ M), inositol 1,4,5-trisphosphate ($10^{-3}$ M), or calmodulin (5 μg/ml) which is an intracellular signaling factor. The present study demonstrates that regucalcin can activate $Ca^{2+}$ pump activity in rat heart microsomes, and that the protein may act the SH groups of $Ca^{2+}$-ATPase by binding to microsomal membranes. J. Cell. Biochem. 86: 184–193, 2002. 

**Key words:** regucalcin; $Ca^{2+}$-ATPase; $Ca^{2+}$ pump; sarcoplasmic reticulum; rat heart

Calcium ion ($Ca^{2+}$) plays an important role in the regulation of many cell functions. Regucalcin was found as a $Ca^{2+}$-binding protein that does not contain the EF-hand motif as a $Ca^{2+}$-binding domain [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1988; Shimokawa and Yamaguchi, 1993]. The gene of regucalcin is localized on the proximal end of rat chromosome Xq 11.1–12 [Shimokawa et al., 1995], and it consists of seven exons and six introns, with several consensus regulatory elements upstream of the 5′-flanking region [Yamaguchi et al., 1996; Murata and Yamaguchi, 1998, 1999; Misawa and Yamaguchi, 2000a]. The gene of regucalcin is highly conserved in vertebrate species [Misawa and Yamaguchi, 2000b]. Regucalcin is mainly present in liver and kidney cortex of rats [Yamaguchi and Isogai, 1993]. Regucalcin mRNA expression is stimulated through $Ca^{2+}$-dependeny signaling mechanism [Misawa and Yamaguchi, 2000a].

Regucalcin may have a multifunctional role in cells [Yamaguchi, 2000a,b; in Review]. Regucalcin has been demonstrated to have an inhibitory effect on $Ca^{2+}$-dependent protein kinases and protein phosphatases in liver and kidney cortex cells [Katsumata and Yamaguchi, 1998; Omura and Yamaguchi, 1998; Yamaguchi and Katsumata, 1999; Morooka and Yamaguchi, 2001], suggesting a regulatory role in $Ca^{2+}$ signaling. Regucalcin has been also shown to suppress cell proliferation of regenerating rat

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liver and cloned hepatoma cells by inhibiting deoxyribonucleic acid synthesis in the nucleus [Yamaguchi and Kanayama, 1996; Inagaki and Yamaguchi, 2001; Misawa et al., 2002]. Regucalcin may be able to suppress overexpression of cell proliferation.

Moreover, regucalcin has been shown to stimulate $Ca^{2+}$ pump activity in the plasma membraneas [Takahashi and Yamaguchi, 1994, 1997; Kurota and Yamaguchi, 1997a], microsomes [Kurota and Yamaguchi, 1997b; Takahashi and Yamaguchi, 1999], and mitochondria [Takahashi and Yamaguchi, 2000; Xue et al., 2000] in rat liver and kidney cortex cells, suggesting that regucalcin plays a role in the regulation of intracellular $Ca^{2+}$ homeostasis. Regucalcin may play a pivotal role in the control of $Ca^{2+}$ concentration in liver and kidney cortex cells.

The $Ca^{2+}$ current is one of the most important components in cardiac excitation–contraction coupling. This coupling mechanism is based on the regulation of intracellular $Ca^{2+}$ concentration by $Ca^{2+}$ pump in the sarcoplasmic reticulum of heart muscle [Fleischer and Inui, 1989; Langer, 1992]. The role of regucalcin of heart muscle function has not been clarified so far. Regucalcin is only slightly present in rat heart [Yamaguchi and Isogai, 1993], although whether regucalcin mRNA is expressed in heart muscle is unknown.

The present study, therefore, was undertaken to determine whether regucalcin could regulate $Ca^{2+}$ pump enzyme ($Ca^{2+}$-ATPase) in the microsomes (sarcoplasmic reticulum) of rat heart muscle. We found that regucalcin, which is expressed in rat heart, had an activatory effect on $Ca^{2+}$-ATPase in the micosomes of rat heart.

## MATERIALS AND METHODS

### Chemicals

Adenosine-5′-triphosphate (ATP), thapsigargin (TP), *N*-ethylmaleimide (NEM), dithiothreitol (DTT), digitonin, dibutyryl cyclic adenosine-5′-monophosphate (DcAMP), inositol 1,4,5-trisphosphate (IP3), and calmodulin (52,000 U/mg protein from bovine brain) were purchased from Sigma Chemical Co. (St. Louis, MO). Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe was purchased from Clontech (Polo Alto, CA). Tag DNA polymerase was obtained from Takara Shuzo Co. (Shiga, Japan). NHS-LC-biotin was obtained from Pierce (Rockford, IL). Streptavidin-peroxidase conjugate was obtained from Tag, Inc. (Burlingame, CA). [$^{45}$Ca] calcium chloride (12.4 GBq/mg) was obtained from New England Nuclear (Boston, MA). Calcium chloride, ethylenglycol-bis-aminoethylether *N*, *N*, *N*′, *N*′ tetracetic acid (EGTA), and other chemicals were purchased from Wako Pure Chemical Company (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, weighting 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% calcium, and 1.1% P at 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]. Protein concentration was determined by the method of Lowry et al. [1951].

### Preparation of Heart Muscle Microsomes

Rats were killed by cardiac puncture. The heart muscle was removed, rinsed with ice-cold 250 mM sucrose solution, cut into small pieces, suspended 1:9 in the homogenization medium containing 250 mM sucrose, 10 mM Tris-HCl, 1.0 mM EGTA, and 1 mM DTT, pH 7.0, and homogenized in a Potter-Elvehjem homogenizer

with a Teflon pestle. The homogenate was centrifuged at 1,000*g* for 10 min to remove nuclei, unbroken cells, and cell debris. The resultant supernatant was centrifuged at 7,700*g* for 20 min to remove the mitochondrial fraction. The postmitochondrial supernatant was then centrifuged at 110,000*g* for 60 min to sediment the microsomal (sarcoplasmic reticulum) fraction. The microsomal fraction was resuspended in buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, and 120 mM KCl, pH 6.8, to a final protein concentration of 1.0–2.0 mg/ml. Protein concentration was determined by the method of Lowry et al. [1951].

### Quantification of Specific mRNA by Reverse Transcription-Polymerase Chain Reaction

Total RNAs were prepared as described previously [Chonczyshi and Sacchi, 1987]. Liver, heart, and brain tissues of young rats were quickly removed, rinsed with ice-cold 250 mM sucrose solution, and homogenized in buffer solution containing 4 M quanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl alcohol, and the phases were separated by centrifugation at 10,000*g* for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isopropanol at −20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate (DEPC)-treated water.

Reverse transcription (RT) was performed with a 4 μg of total RNA and random hexamers as primers using a commercial system (Superscript Preamplification System, Life Technologies). After RT, the reaction contents were digested with RNase H, and purified by phenol/chloroform extraction and ethanol precipitation. The cDNA products were dissolved in 50 μg of DEPC-treated water. Primers for amplification of regucalcin cDNA were: 5′-AGATGAACAAATCCCAGAT-3′ (sense strand, positions 618–636 of cDNA sequence in reference [Misawa and Yamaguchi, 2000b] and 5′-TCACCCTGCATAGGAATAT-3′ (antisense strand, positions 924–906 of cDNA sequence [Misawa and Yamaguchi, 2000b]). These oligonucleotid sequences are localized in exon 5 and 7, respectively, of the regucalcin gene. The pair of oligonucleotide primers was designed to amplify a 307-bp sequence from the mRNA of mouse regucalcin. For quantitative PCR, G3PDH was used as an internal control to evaluate total RNA input. Primers for amplification of G3PDH cDNA were: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (sense strand) and 5′-CATGTAGGCCATGAGGTCCACCAC3′ (antisense strand) from the G3PDH Amplimer Set (Clontech). Polymerase chain reaction (PCR) was performed by using reaction mixture (20 μl) containing 1 μl of tissue specific cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate, 20 pmol of primers, 10% glycerol, 2.5 U Taq DNA polymerase. Samples were amplified for 30 cycles under the following conditions; denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C.

The signals of the PCR products were analyzed by Southern blot technique [Southern, 1975]. The amplified products were separated by electrophosresis on a 2% agarose gel and then transferred onto a nylon membrane. To demonstrate that the bands obtained by reverse transcription-polymerase chain reaction (RT-PCR) were derived from regucalcin mRNA or G3PDH mRNA, regucalcin cDNA (positions 618–924 of cDNA sequence in reference [Misawa and Yamaguchi, 2000]) and G3PDH cDNA were used as a hybridization probe. These probes were labeled with deoxycytidine 5′-[α-$^{32}$P] triphosphate ([$^{32}$P]dCTP; 110 Tbq/mmol), obtained from Amersham (Bucking Ramshire, UK), by random primers and DNA polymerase Klenow fragment [Feinberg and Vogelsrtein, 1983]. The membranes were incubated in a prehybridization buffer containing 50% formamide, 5 × SSPE, 5 × Denhardt's reagent, 0.5% SDS, and 0.1 mg/ml denatured with $^{32}$P-labeled regucalcin cDNA probe of G3PDH cDNA probe in the same buffer at 42°C for 20 min, once 1 × SSPE and 0.1% SDS at 42°C for 1 h, and twice with 0.1 × SSPE and 0.1% SDS at room temperature for 20 min. The blots were autoradiographed on X-ray film with intensifying screens for visualization of the amplification products.

### Western Blot Analysis

The homoganate from heart muscle tissues was centrifuged at 7,700*g* for 20 min, and the supernatant was centrifuged for 1 h at 110,000*g* at 4°C, and the supernatant (cytosol) and pellet (microsomes) were used for Western blot analysis [Wesssendorf et al., 1993]. Aliquots of cytosol (25 μg of protein) and microsomes (25 μg of protein) were mixed with 5 × Laemmli sample buffer, boiled for 5 min, and SDS–PAGE

was performed by the method of Laemmli [1990] using 12% polyacrylamide resolving gel. After SDS–PAGE, the proteins were then transferred onto a polyvinrylidene difluoride membrane at 100 mA for 4 h. The membranes were incubated with a polyclonal rabbit anti-regucalcin antibody [Yamaguchi and Isogai, 1993], which was diluted 1:2000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% (w/v) Tween-20 (washing buffer), and 5% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horseradish peroxidase linked anti-rabbit IgG which was diluted 1:5000 with washing buffer containing 5% (w/v) skim milk, and again they were washed. Detection of the protein bands was performed using an enhanced chemiluminescent kit following the manufacture's instructions. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel. The membranes were exposed for 15 min of the film.

### Assay of $Ca^{2+}$-ATPase

$Ca^{2+}$-ATPase activity in the microsomes of heart muscle was assayed by the procedure of Narayanan et al. [1982]. $Mg^{2+}$-ATPase activity was determined for 10 min at 37°C in a medium (1.0 ml) containing 50 mM Tris-HCl buffer (pH 6.8), 5 mM $MgCl_2$, 120 mM KCl, 5 mM $NaN_3$, 0.2 mM EGTA, 2.5 mM Mg-ATP (neutralized with KOH), and the microsomes (100–200 μg as protein) in the absence of $CaCl_2$. $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured in the same medium containing 10 μM $CaCl_2$ without EGTA addition. The enzyme reaction was stopped by the addition of 10% trichloroacetic acid (1.0 ml). The amount of inorganic phosphate released from ATP by enzyme reaction was measured according to the method of Nakamura and Mori [1958]. $Ca^{2+}$-ATPase activity was calculated as the difference between $(Ca^{2+} + Mg^{2+})$-ATPase and $Mg^{2+}$-ATPase. Enzyme activity was expressed as nanomole of inorganic phosphate released per minute per milligram (mg) protein.

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

A $^{45}Ca^{2+}$ uptake by the microsomes of heart muscle was measured by the Millipore filtration technique [Moore and Kraus-Friedmann, 1983]. About 100–150 μg of protein/ml reaction of mixture was incubated for 15 min 37°C in 1 ml of medium containing 100 mM KCl, 20 mM HEPES, 1 mM $NaN_3$, 1 mM $MgCl_2$, 1 μM ruthenium red, and100 μM $CaCl_2$ containing $^{45}Ca^{2+}$ (0.185 MBq), pH 6.8. At a designated time after the addition of 10 mM adenosine trisphosphate (ATP), adjusted to pH 6.8 with KOH, to initiate energy-dependent $Ca^{2+}$ uptake, a 100-μl sample was filtered through a 0.22-μm pre-wetted Millipore filter. The precipitate was washed with 120 mM KCl/100 mM HEPES, pH 6.8, transferred to a scintillation vial and counted for radioactivity. $^{45}Ca^{2+}$ uptake is expressed as nanomole of $^{45}Ca^{2+}$ accumulated per milligram protein of the microsomes.

### 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

### Expression of Regucalcin in Heart Muscle

The expression of regucalcin mRNA was examined by RT-PCR in the liver, brain, and heart muscle of rats using regucalcin-specific primers. The housekeeping gene *G3PDH* was used as an internal control. The amount of each cDNA sample included in the reaction mixture was adjusted to the same quantity according to the G3PDH mRNA expression. The autoradiography of Southern blots as RT-PCR products was shown in Figure 1. Southern blot analysis demonstrated that the 307-bp bands obtained by RT-PCR corresponded to the region between regucalcin-specific primers. A strong signal was seen in liver and also weak signals were observed in brain and heart, indicating that regucalcin mRNA is expressed in heart muscle as well as in liver and brain.

Regucalcin protein levels in rat heart muscle were analyzed by Western blotting (Fig. 2). A polyclonal rabbit anti-regucalcin antibody was used [Yamaguchi and Isogai, 1993]. Regucalcin has been found to be 33,388 Da [Shimokawa and Yamaguchi, 1993]. A single immunoreactive band migrating identically as regucalcin was detected by immunoblotting after electrophoresis of the cytosol of homogenate obtained from rat heart muscle. The band, however, was not detected in the microsomes obtained from the homogenate of rat heart muscle.

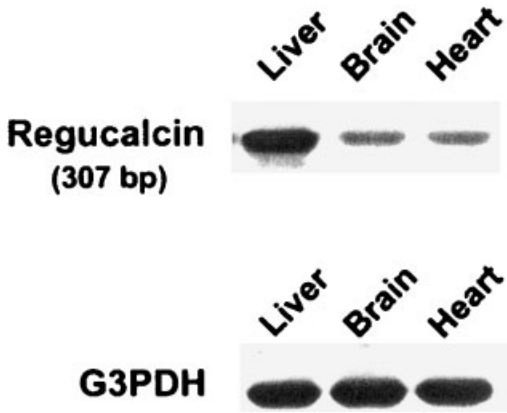


**Fig. 1.** Detection of regucalcin mRNA in rat heart muscle by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNAs isolated from the indicated rat tissues were analyzed by RT-PCR. Rat regucalcin-specific primers were designed to amplify a 307-bp fragment of regucalcin. The housekeeping gene *G3PDH* was used as an internal control. The signals of the amplification products were analyzed by Southern blotting. The amount of each cDNA sample included in the reaction was adjusted to the same quantity according to the G3PDH mRNA expression. The figure shows one of the three experiments with separate samples.

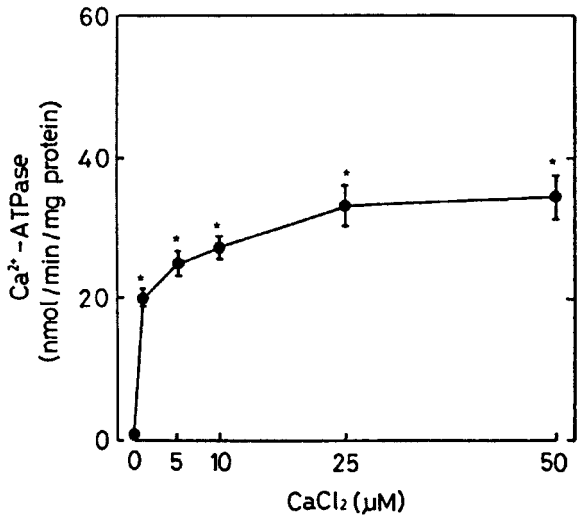


**Fig. 3.** Alteration in $Ca^{2+}$-ATPase activity with increasing concentrations of $Ca^{2+}$ addition in the microsomes of rat heart muscle. $CaCl_2$ was added to the enzyme reaction mixture, yielding concentrations of 1, 5, 10, 25, and 50 μM. Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, as compared with the control value without $Ca^{2+}$ addition.

### Effect of Regucalcin on $Ca^{2+}$ Uptake Activity by Heart Muscle Microsomes

The effect of calcium chloride addition on $Ca^{2+}$-ATPase activity in rat heart microsomes (sarcoplasmic reticulum) is shown in Figure 3. The addition of calcium chloride (1, 5, 10, 25, and 50 μM) in the enzyme reaction mixture led to a significant increases in $Ca^{2+}$-ATPase activity; the increase was saturated at 10–25 μM. In the presence of 10 μM $CaCl_2$, the addition of regucalcin ($10^{-10}$–$10^{-8}$ M) produced a significant elevation in $Ca^{2+}$-ATPase activity. The effect of regucalcin reached to a maximum at the concentration of $10^{-8}$ M (Fig. 4A). Meanwhile, heart microsomal $Mg^{2+}$-ATPase activity was not appreciably altered by the addition of regucalcin ($10^{-11}$–$10^{-8}$ M) (Fig. 4B).

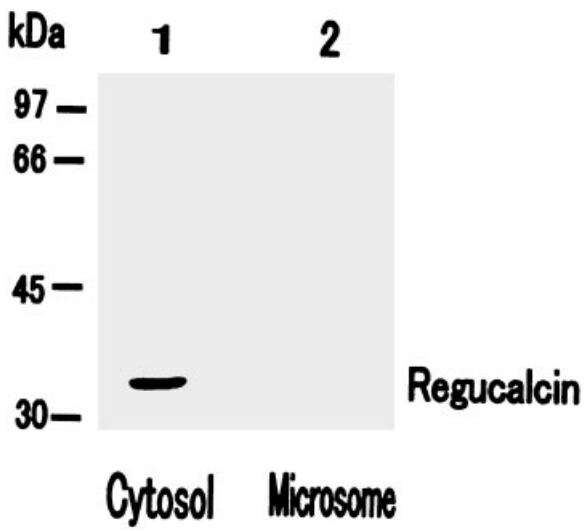


**Fig. 2.** Detection of regucalcin protein in the cytosol of rat heart muscle. Western blot analysis was carried out on the extracts (25 μg of cytosolic protein) obtained from the heart muscle of rats. **Lane 1**, cytosol; **lane 2**, microsomes. The figure shows one of the four experiments with separate sample.

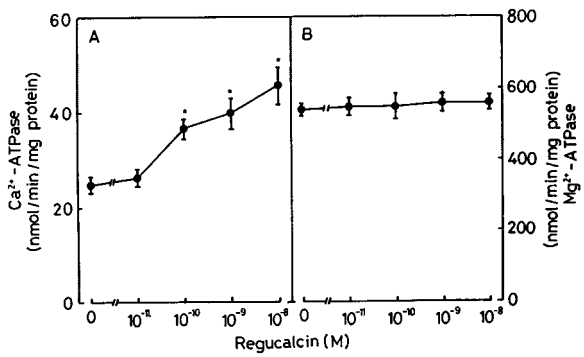


**Fig. 4.** Effect of regucalcin on $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase activities in the microsomes of rat heart muscle. Regucalcin was added to the enzyme reaction mixture, yielding concentrations of $10^{-11}$–$10^{-8}$ M in the presence (**A**) or absence (**B**) of 10 μM $CaCl_2$. Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, as compared with the control value without regucalcin addition.

TP is a specific inhibitor of the microsomal $Ca^{2+}$ pump enzyme ($Ca^{2+}$-ATPase) [Thastrup et al., 1990]. Heart microsomal $Ca^{2+}$-ATPase activity was markedly decreased by the addition of TP in the enzyme reaction mixture in the presence of calcium chloride (10 μM) (Fig. 5). The inhibitory effect of TP was saturated at $10^{-6}$ M (Fig. 5A). The effect of regucalcin ($10^{-8}$ M) increasing $Ca^{2+}$-ATPase activity was entirely seen in the presence of TP ($10^{-5}$ M) (Fig. 5B), indicating that regucalcin acts $Ca^{2+}$ pump enzyme ($Ca^{2+}$-ATPase) in the heart microsomes.

The effect of regucalcin on ATP-dependent $^{45}Ca^{2+}$ uptake by the microsomes of heart muscle is shown in Figure 6. The addition of ATP to the reaction mixture containing $^{45}Ca^{2+}$ led to microsomal $^{45}Ca^{2+}$ uptake (Fig. 6A). A significant increase in microsomal $^{45}Ca^{2+}$ uptake by regucalcin was seen at $10^{-10}$ M, and it was saturated at $10^{-8}$M (Fig. 6B). Thus, regucalcin had an activatory effect on $Ca^{2+}$ pump in the microsomes of rat heart muscle.

## Characterization of Regucalcin Action on Heart Microsomal $Ca^{2+}$-ATPase Activity

The effect of digitonin on regucalcin-increased $Ca^{2+}$-ATPase activity in rat heart microsomes is shown in Figure 7. Digitonin has a solubilization effect on membranous lipid [Murphy et al., 1980]. The presence of digitonin ($10^{-3}$ and $10^{-2}$%) in the enzyme reaction mixture with the addition of calcium chloride (10 μM) caused a significant decrease in $Ca^{2+}$-ATPase activity. In the presence of digitonin, regucalcin could not increase $Ca^{2+}$-ATPase activity.

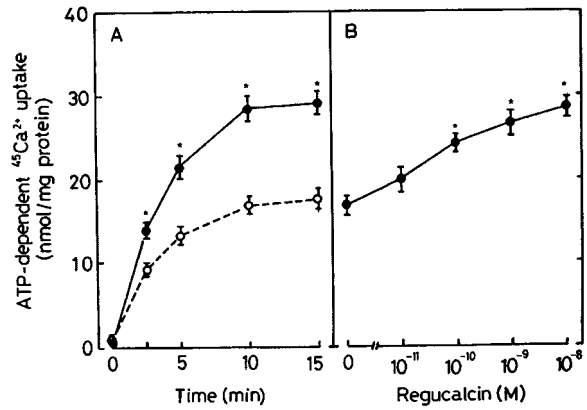


**Fig. 6.** Effect of regucalcin on ATP-dependent $^{45}Ca^{2+}$ uptake in the microsomes of rat heart muscle. $^{45}Ca^{2+}$ uptake was measured as described in the experimental section. **A**: The microsomes were incubated for 2.5, 5, 10, and 15 min after the addition of adenosine-5′-triphosphate (ATP) in the absence or presence of regucalcin ($10^{-8}$ M). **B**: The microsomes were incubated for 10 min after the addition of ATP in the absence or presence of regucalcin ($10^{-4}$–$10^{-8}$ M). Each value is the mean ± SEM of five experiments with separate rats. $^{*}P < 0.01$, as compared with the control (none) value; white bars, control (none); black bars, regucalcin.

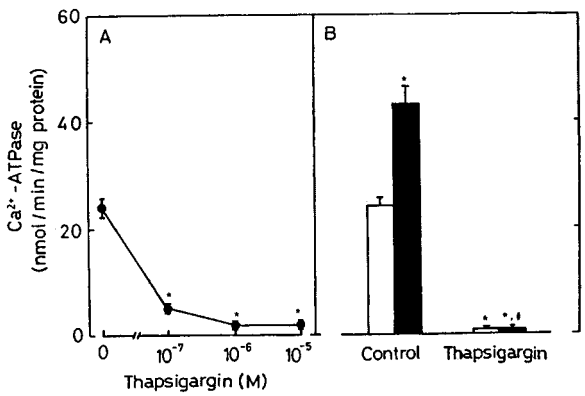


**Fig. 5.** Effect of thapsigargin (TP), an inhibitor of sarcoplasmic reticulum $Ca^{2+}$-ATPase, on regucalcin-increased $Ca^{2+}$-ATPase activity in the microsomes of rat heart muscle. **A**: TP was added to the enzyme reaction mixture, yielding concentrations of $10^{-7}$–$10^{-5}$ M. **B**: The enzyme reaction mixture contained either vehicle or regucalcin ($10^{-8}$ M) in the absence or presence of TP ($10^{-5}$ M). Each value is the mean ± SEM of five experiments with separate rats. $^{*}P < 0.01$, as compared with the control (none) value; $\#P < 0.01$, as compared with the value of regucalcin alone. White bars, control; black bars, regucalcin.

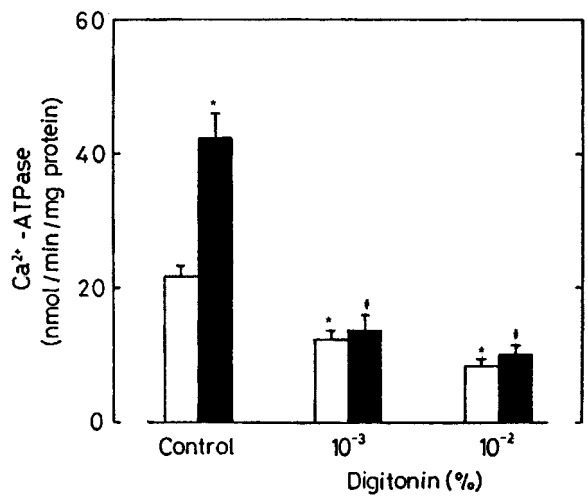


**Fig. 7.** Effect of digitonin on regucalcin-increased $Ca^{2+}$-ATPase activity in the microsomes of rat heart muscle. The enzyme reaction mixture contained either vehicle or digitonin ($10^{-3}$ or $10^{-2}$%) in the absence or presence of regucalcin ($10^{-8}$ M). Each value is the mean ± SEM of five experiments with separate rats. $^{*}P < 0.01$, as compared with the control (none) value. $\#P < 0.01$, as compared with the value of regucalcin alone. White bars, control; black bars, regucalcin.

The effect of NEM, a modifying reagent of sulfhydryl (SH) groups, or DTT, a protecting reagent of SH groups, on regucalcin-increased $Ca^{2+}$-ATPase activity in heart microsomes is shown in Figure 8. The presence of NEM (5 mM) in the enzyme reaction mixture did not have a significant effect on the enzyme activity. In the presence of NEM (5 mM), however, the effect of regucalcin ($10^{-8}$ M) in increasing $Ca^{2+}$-ATPase activity was not seen (Fig. 8). Meanwhile, the presence of DTT (5 mM) in the enzyme reaction mixture produced a remarkable elevation of $Ca^{2+}$-ATPase activity (Fig. 8). The presence of regucalcin ($10^{-8}$ M) did not significantly enhance the enzyme activity.

Vanadate has an inhibitory effect on phosphorylation of enzyme by ATP [Chen and Junger, 1983]. The presence of vanadate (1 mM) in the enzyme reaction mixture did not have a significant effect on heart microsomal $Ca^{2+}$-ATPase activity (Fig. 8). In the presence of vanadate (1 mM), however, the effect of regucalcin in increasing $Ca^{2+}$-ATPase activity was completely abolished.

### Effect of Signaling Factors on Regucalcin-Increased Heart Microsomal $Ca^{2+}$-ATPase Activity

The effect of DcAMP on $Ca^{2+}$-ATPase activity in rat heart microsomes is shown in Figure 9. $Ca^{2+}$-ATPase activity was significantly increased in the presence of DcAMP ($10^{-6}$–$10^{-4}$ M) in the enzyme reaction mixture with the addition of calcium chloride (10 μM) (Fig. 9A). This increase was not significantly enhanced in the presence of regucalcin ($10^{-8}$ M) (Fig. 9B).

The presence of inositol 1,4,5-trisphosphate (IP3; $10^{-5}$–$10^{-3}$ M) in the enzyme reaction mixture did not have a significant effect on heart microsomal $Ca^{2+}$-ATPase activity (Fig. 10A).

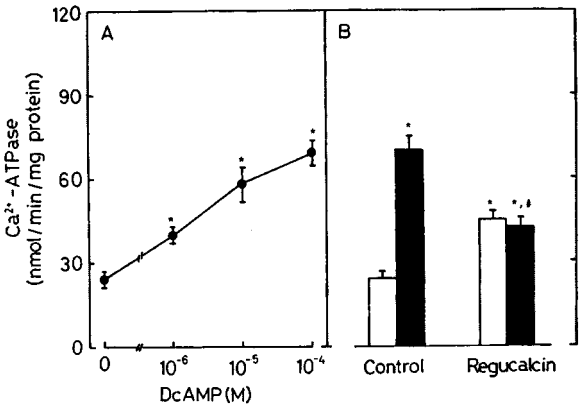


**Fig. 9.** Effect of dibutyryl cyclic adenosine monophosphate (DcAMP) on regucalcin-increased $Ca^{2+}$-ATPase activity in the microsomes of rat heart muscle. **A**: DcAMP was added to the enzyme reaction mixture, yielding concentrations of $10^{-6}$–$10^{-4}$ M. **B**: The enzyme reaction mixture contained either vehicle or DcAMP ($10^{-4}$ M) in the absence or presence of regucalcin ($10^{-8}$ M). Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, as compared with the control (none) value; $\#P < 0.01$, as compared with the value of DcAMP alone. White bars, without DcAMP; black bars, with DcAMP.

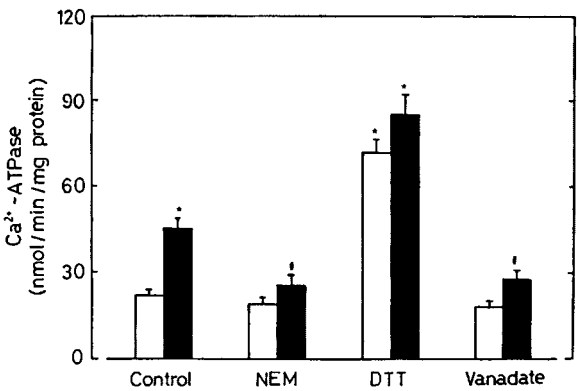


**Fig. 8.** Effect of *N*-ethylmaleimide (NEM), dithiothreitol (DTT) or vanadate on regucalcin-increased $Ca^{2+}$-ATPase activity in the microsomes of rat heart muscle. The enzyme reaction mixture contained either vehicle, NEM (5 mM), DTT (5 mM) or vanadate (0.1 mM) in the absence or presence of regucalcin ($10^{-8}$ M). Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, as compared with the control (none) value; $\#P < 0.01$, as compared with the value of regucalcin alone. White bars, control; black bars, regucalcin.

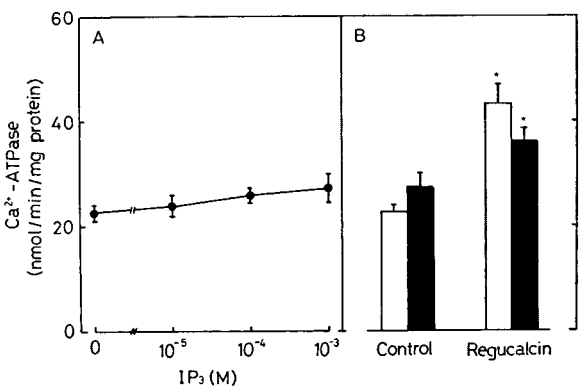


**Fig. 10.** Effect of inositol 1,4,5-trisphoaphate (IP3) on regucalcin-increased $Ca^{2+}$-ATPase activity in the microsomes of rat heart muscle. **A**: IP3 was added to the enzyme reaction mixture, yielding concentrations of $10^{-5}$–$10^{-3}$ M. **B**: The enzyme reaction mixture contained either vehicle or IP3 ($10^{-3}$ M) in the absence or presence of regucalcin ($10^{-8}$ M). Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, as compared with the control (none) value; white bars, without IP3; black bars, with IP3.

The effect of regucalcin ($10^{-8}$ M) in increasing $Ca^{2+}$-ATPase activity was not significantly altered in the presence of IP3 ($10^{-3}$ M) (Fig. 10B).

Heart microsomal $Ca^{2+}$-ATPase activity was not significantly altered by the addition of calmodulin (1, 2.5, and 5 μg/ml) in the enzyme reaction mixture (Fig. 11A). The effect of regucalcin ($10^{-8}$ M) on $Ca^{2+}$-ATPase activity was not significantly altered by the addition of calmodulin (5 μg/ml) in the presence of calcium chloride (10 μM) (Fig. 11B).

## DISCUSSION

The role of regucalcin in the regulation of heart muscle function has not clarified so far. Regucalcin has been shown to be $3.86 \times 10^{-8}$ M in the tissues of rat heart muscle [Yamaguchi and Isogai, 1993]. The present study demonstrates that regucalcin mRNA is expressed in rat heart muscle using RT-PCR analysis. The result with Western blot analysis indicates that regucalcin is present in the cytoplasm of heart muscle cells. The present study, moreover, was undertaken to determine the effect of regucalcin on $Ca^{2+}$ pump activity in the microsomes (sarcoplasmic reticulum) of rat heart muscle, which regulates intracellular $Ca^{2+}$ concentration related to cardiac excitation–contraction coupling [Fleischer and Inui, 1989; Langer, 1992]. Regucalcin was found to increase $Ca^{2+}$-ATPase activity and ATP-dependent $Ca^{2+}$ uptake in rat heart microsomes, suggesting its role in the regulation of heart muscle function.

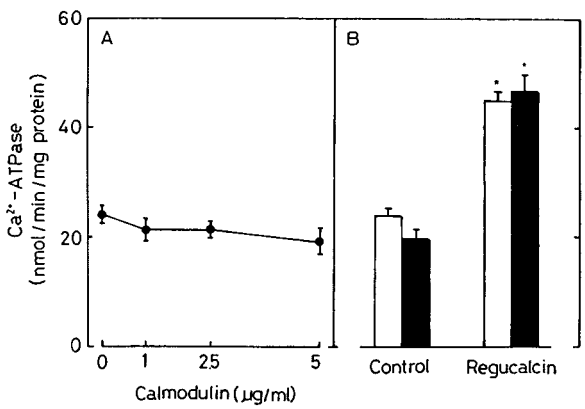


**Fig. 11.** Effect of calmodulin on regucalcin-increased $Ca^{2+}$-ATPase activity in the microsomes of rat heart muscle. **A**: Calmodulin was added to the enzyme reaction mixture, yielding concentrations of 1, 2.5, and 5 μg/ml. **B**: The enzyme reaction mixture contained either vehicle or calmodulin (5 μg/ml) in the absence or presence of regucalcin ($10^{-8}$ M). Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, as compared with the control (none) value; white bars, without calmodulin; black bars, with calmodulin.

$Ca^{2+}$-ATPase activity in the microsomes (sarcoplasmic reticulum) of rat heart muscle was markedly decreased in the presence of TP, a specific inhibitor of the microsomal $Ca^{2+}$ pump enzyme ($Ca^{2+}$-ATPase) [Thastrup et al., 1990]. The effect of regucalcin in increasing microsomal $Ca^{2+}$-ATPase activity was not seen in the presence of TP, indicating that regucalcin activates microsomal $Ca^{2+}$ pump enzyme. The effect of regucalcin on $Ca^{2+}$-ATPase activity was completely prevented 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 acts on $Ca^{2+}$-ATPase by its binding on the microsomal membranous lipids. Moreover, regucalcin-increased microsomal $Ca^{2+}$-ATPase activity was completely inhibited in the presence of NEM, a modifying reagent of SH groups, while the effect of regucalcin was not further enhanced by the addition of DTT, a protecting reagent of SH groups. It has been reported that the microsomal $Ca^{2+}$ sequestration is critically dependent on the SH groups of protein, and that modification of protein thiols may be an important mechanism for the inhibition of microsomal $Ca^{2+}$ sequestration by a variety of toxic agents [Thor et al., 1985]. Presumably, regucalcin binds to the lipids at the close site of $Ca^{2+}$-ATPase in heart microsomes, and regucalcin acts on the SH group which may be an active site of the enzyme.

Vanadate is an inhibitor of phosphorylation of $Ca^{2+}$-ATPase by ATP [Chen and Junger, 1983]. The effect of regucalcin in increasing heart microsomal $Ca^{2+}$-ATPase activity was completely blocked in the presence of vanadate. The phosphorylated intermediate of the $Ca^{2+}$ pump enzyme is associated with an ATP-dependent $Ca^{2+}$ transport system [Chen and Junger, 1983]. Regucalcin may stimulate $Ca^{2+}$-dependent phosphorylation of $Ca^{2+}$-ATPase in heart muscle microsomes.

Cyclic adenosine monophosphate (cAMP) and IP3 are intracellular signaling factors [Rasmussen, 1970; Berridge and Irvine, 1989]. It is known that cAMP and IP3 can stimulate $Ca^{2+}$ release from the microsomes [Fleischer and Inui, 1989; Joseph and Williamson, 1989; Endoh, 1991]. The presence of DcAMP in the enzyme reaction mixture caused a significant

increase in heart microsomal $Ca^{2+}$-ATPase activity, whereas IP3 did not have an effect on the enzyme activity. cAMP may have a role in the regulation of $Ca^{2+}$-ATPase activity due to hormonal stimulation [Endoh, 1991]. The effect of regucalcin in increasing microsomal $Ca^{2+}$-ATPase activity was not modulated in the presence of DcAMP or IP3 in the reaction mixture. Meanwhile, calmodulin did not have a significant effect on heart microsomal $Ca^{2+}$-ATPase activity. The effect of regucalcin on $Ca^{2+}$-ATPase activity was seen in the presence of calmodulin. Presumably, regucalcin is unique as an activator of $Ca^{2+}$-ATPase in rat heart microsomes.

Phospholamban is known to regulate $Ca^{2+}$ pump enzyme ($Ca^{2+}$-ATPase) in the sarcoplasmic reticulum of heart muscle [Tada and Kadoma, 1989]. $Ca^{2+}$-ATPase is activated by cAMP-dependent phosphorylation of phospholamban following hormonal stimulation [Tada and Kadoma, 1989]. At present, the endogenous activatory protein of sarcoplasmic reticulum $Ca^{2+}$-ATPase may be unknown. Regucalcin, which is present in the cytoplasm of heart muscle, may play an important role as an endogenous activator in the regulation of sarcoplasmic reticulum $Ca^{2+}$-ATPase activity in rat heart muscle. The effect of regucalcin to activate sarcoplasmic reticulum $Ca^{2+}$-ATPase was seen in the range of $10^{-10}$–$10^{-8}$ M of regucalcin addition. Regucalcin content in rat heart muscle tissues was $3.86 \times 10^{-8}$ M [Yamaguchi and Isogai, 1993]. Presumably, regucalcin with a physiologic concentration can activate sarcoplasmic reticulum $Ca^{2+}$-ATPase in rat heart muscle. Regucalcin may play a physiologic role in the regulation of cardiac excitation–contraction coupling.

In conclusion, it has been demonstrated that regucalcin is expressed in rat heart muscle, and that the protein can activate $Ca^{2+}$-ATPase ($Ca^{2+}$ pump enzyme) in the sarcoplasmic reticulum.

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