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Regucalcin Increases Ca^{2+}-ATPase Activity in the Mitochondria of Brain Tissues of Normal and Transgenic Rats

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Abstract The role of regucalcin, which is a regulatory protein in intracellular signaling, in the regulation of Ca^{2+}-ATPase activity in the mitochondria of brain tissues was investigated. The addition of regucalcin (10^{-10} to 10^{-8} M), which is a physiologic concentration in rat brain tissues, into the enzyme reaction mixture containing 25 μM calcium chloride caused a significant increase in Ca^{2+}-ATPase activity, while it did not significantly change in Mg^{2+}-ATPase activity. The effect of regucalcin (10^{-9} M) in increasing mitochondrial Ca^{2+}-ATPase activity was completely inhibited in the presence of ruthenium red (10^{-7} M) or lanthanum chloride (10^{-7} M), both of which are inhibitors of mitochondrial uniporter activity. Whether the effect of regucalcin is modulated in the presence of calmodulin or dibutyryl cyclic AMP (DcAMP) was examined. The effect of regucalcin (10^{-9} M) in increasing Ca^{2+}-ATPase activity was not significantly enhanced in the presence of calmodulin (2.5 μg/ml) which significantly increased the enzyme activity. DcAMP (10^{-6} to 10^{-4} M) did not have a significant effect on Ca^{2+}-ATPase activity. The effect of regucalcin (10^{-9} M) in increasing Ca^{2+}-ATPase activity was not seen in the presence of DcAMP (10^{-4} M). Regucalcin levels were significantly increased in the brain tissues or the mitochondria obtained from regucalcin transgenic (RC TG) rats. The mitochondrial Ca^{2+}-ATPase activity was significantly increased in RC TG rats as compared with that of wild-type rats. This study demonstrates that regucalcin has a role in the regulation of Ca^{2+}-ATPase activity in the brain mitochondria of rats. J. Cell. Biochem. 104: 795–804, 2008.

Key words: regucalcin; Ca^{2+}-ATPase; calcium; mitochondria; brain; regucalcin transgenic rat

Regucalcin was discovered in 1978 as a calcium-binding protein that does not contain the EF-hand motif of the Ca^{2+}-binding domain [Yamaguchi and Yamamoto, 1978; Yamaguchi and Sugii, 1981; Yamaguchi, 1988]. The name regucalcin was proposed for this Ca^{2+}-binding protein, which regulates the Ca^{2+} effect on liver cell function [Yamaguchi and Mori, 1988; Yamaguchi, 1992; Shimokawa and Yamaguchi, 1993].

The gene of regucalcin is highly conserved in vertebrate species [Misawa and Yamaguchi, 2000]. The human and rat regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. Regucalcin is greatly expressed in liver and kidney cortex, and the expression is mediated through Ca^{2+}-signaling mechanism due to hormonal stimulation [Yamaguchi and Isogai, 1993; Murata and Yamaguchi, 1999; Nakagawa and Yamaguchi, 2005a]. AP1, NF1-A1, and RGPR-p117 have been found to enhance transcriptional activity [Murata and Yamaguchi, 1998; Misawa and Yamaguchi, 2002; Sawada and Yamaguchi, 2006]. The expression of regucalcin mRNA is mediated through a Ca^{2+}-dependent signaling mechanism [Shimokawa and Yamaguchi, 1993; Murata and Yamaguchi, 1999].

Regucalcin is greatly localized in liver, and its relatively higher levels are also found in kidney cortex of rats [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. Recent study has demonstrated that regucalcin plays a multifunctional role as a regulatory protein on intracellular signaling process in the cytoplasm and nucleus of cells [Yamaguchi, 2000a,b in reviews]. Regucalcin has been shown to play a

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role in the maintenance of intracellular Ca^{2+} homeostasis and in the inhibitory regulation of various Ca^{2+}-dependent protein kinases, tyrosine kinases, and protein phosphatases, nitric oxide synthase, and in the suppression of nuclear DNA and RNA syntheses [Yamaguchi, 2000a,b, 2005]. Regucalcin has also been demonstrated to have suppressive effects on cell proliferation, cell death, and apoptosis induced by various factors [Misawa et al., 2002; Izumi and Yamaguchi, 2004; Nakagawa and Yamaguchi, 2005b]. Regucalcin is located into the nucleus, although the protein is also found in the cytoplasma, microsomes and mitochondria [Tsurusaki et al., 2000]. Regucalcin regulates gene expression in the nucleus [Tsurusaki and Yamaguchi, 2003, 2004]. Thus regucalcin has been proposed to play a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation [Yamaguchi, 2005].

Regucalcin mRNA and its protein are expressed in rat brain tissues and brain neuronal cells [Yamaguchi et al., 1999, 2000]. Regucalcin may play a role in the regulation of brain function. Regucalcin has been shown to have inhibitory effects on Ca^{2+}-dependent protein kinase [Hamano et al., 1999; Hamamo and Yamaguchi, 2001], protein phosphatase [Hamamo and Yamaguchi, 1999], and nitric oxide synthase [Tobisawa and Yamaguchi, 2003] activities in the cytoplasm of rat brain tissues, suggesting its suppressive role in the regulation of brain function.

Regucalcin has been shown to regulate Ca^{2+} homeostasis in liver and kidney cells which the protein is mainly expressed [Yamaguchi, 2005]. The role of regucalcin in the regulation of Ca^{2+} homeostasis in brain tissues has not been fully clarified, although regucalcin has been reported to have inhibitory effects on Ca^{2+}-ATPase activity in the microsomes of rat brain tissues [Yamaguchi et al., 1999].

This study was undertaken to determine whether regucalcin has a role in the regulation of mitochondrial Ca^{2+}-ATPase activity in rat brain tissues. We found that regucalcin can increase mitochondrial Ca^{2+}-ATPase activity in rat brain tissues.

MATERIALS AND METHODS

Chemicals

Adenosine-5′-triphosphate (ATP), ruthenium red, lanthanum chloride ($LaCl_3$), dibutyryl cyclic AMP (DcAMP), and calmodulin (56,500 units/mg protein from bovine brain) were purchased from Sigma Chemical Co. (St. Louis, MO). Calcium chloride and all other chemicals were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Many reagents used were dissolved in distilled water then passed through an ion-exchange resin to remove metal ions.

Animals

Female Wistar rats (4 or 50 weeks old) 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 (w/v) 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 105,00*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 electorophoretic 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 Brain Mitochondria

Rats were killed by cardiac puncture, and the brain removed was washed with ice-cold 250 mM sucrose solution, immediately cut into small pieces, suspended 1:9 in the homogenization medium containing 250 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. (HEPES), and 1.0 mM ethyleneglycol bis(2-amino-ethylether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), pH 7.4, and homogenized in a Potter–Elvehjem homogenizer with a Teflon

pestle [Berthon et al., 1981]. The homogenate was centrifuged at 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 sediment the mitochondrial fraction. The mitochondrial fraction was resuspended in 5 mM $MgCl_2$, 50 mM KCl, and 10 mM HEPES, pH 7.0, to a final protein concentration of 0.7–0.9 mg/ml.

Assay of Ca^{2+}-ATPase

Mg^{2+}-ATPase activity was determined for 10 min at 37°C in a medium containing 10 mM HEPES–KOH buffer (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 6 mM succinate, 8 mM Mg-ATP, and the mitochondria (70–90 μg as protein) in the presence or absence of regucalcin (10^{-11} to 10^{-8} M) [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 25 μM $CaCl_2$ in the presence or absence 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 nanomol of inorganic phosphate released per minute per milligram protein. Protein concentration was determined using the method of Lowry et al. [1951].

Regucalcin Transgenic (RC TG) Rats

RC TG rats (Sprague–Dawley) were generated previously [Yamaguchi et al., 2002]. The TG rats (7 weeks old) were obtained from Japan SLC Inc. (Hamamatsu, Japan). RC TG rats were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% calcium, and 1.1% phosphorus at room temperature (25°C) and were allowed distilled water freely.

Western Blot Analysis

The supernatant after centrifugation at 800*g* for 10 min of homogenate obtained from brain tissues and the mitochondrial fraction were used for Western blot analysis [Wessendolf et al., 1993]. Aliquots of supernatant or mitochondria (40 μg of protein) were mixed with 5× Laemmli sample buffer, boiled for 5 min, and SDS–PAGE was performed using the method of Laemmli [1970] with 12% polyacrylamide resolving gel. After SDS–PAGE, the proteins were transferred onto a polyvinylidene 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 1:2,000 in 10 mM Tris–HCl, pH 8.0, 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 1:5,000 with washing buffer containing 5% (w/v) skim milk. Detection of the protein bands was performed using an enhanced chemiluminescent kit following the manufacturer's instructions. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel. The membranes obtained were exposed for 5 min to film.

Statistical Analysis

Data are expressed as the mean ± SEM. The significance of the difference between the values was estimated by Student's *t*-test. We also used a multiway ANOVA comparison test to compare the treatment groups. A $P < 0.05$ was considered to indicate statistically significant difference.

RESULTS

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

The effect of increasing calcium chloride addition on Ca^{2+}-ATPase activity in the mitochondria of brain tissues obtained from 4 weeks old rats or 50 weeks old rats was examined (Fig. 1). Ca^{2+}-ATPase activity was increased by addition of calcium chloride (5, 10, 25, 50, or 100 μM) into the enzyme reaction mixture. The effect of calcium chloride addition on Ca^{2+}-ATPase activity did not significantly change in the mitochondria obtained from aged rats (50 weeks old). The effect of calcium chloride addition in increasing Ca^{2+}-ATPase activity was saturated with 25 μM, which is closely related to physiologic concentration in the mitochondria.

The effect of regucalcin addition on Ca^{2+}-ATPase activity in the mitochondria of brain tissues obtained from 4 weeks old rats or 50 weeks old rats is shown in Figure 2. Ca^{2+}-ATPase activity in the brain mitochondria from

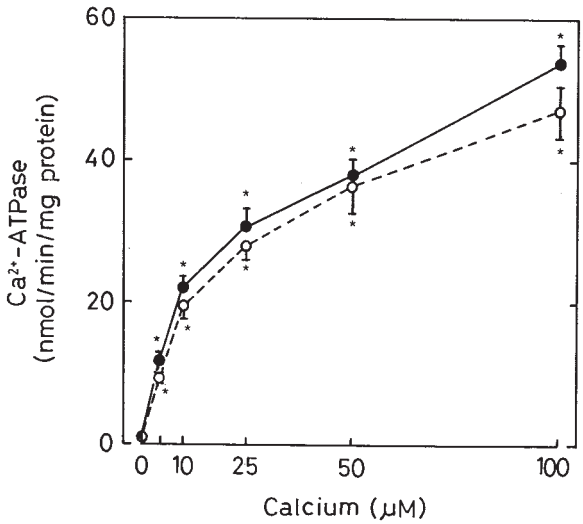

Fig. 1. Effect of increasing calcium chloride addition on Ca^{2+}-ATPase activity in the mitochondria of rat brain tissues. Brain mitochondria was obtained from 4 weeks old rats or 50 weeks old rats. The enzyme reaction mixture contained either vehicle or calcium chloride (5.0–100.0 μM) in the presence of brain mitochondria (70–90 μg of protein/ml of enzyme reaction mixture). Each value is the mean ± SEM of 6–10 experiments with separate mitochondria. $^*P < 0.01$ compared with the value without calcium addition. Open circles, 4 weeks old rats; closed circles, 50 weeks old rats.

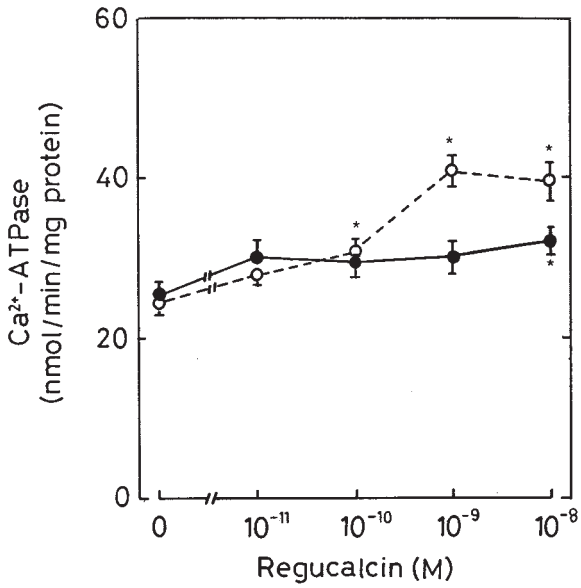

Fig. 2. Effect of regucalcin addition on Ca^{2+}-ATPase activity in the mitochondria of rat brain tissues. Brain mitochondria was obtained from 4 weeks old rats or 50 weeks old rats. The enzyme reaction mixture contained either vehicle or regucalcin (10^{-11} to 10^{-8} M) with or without calcium chloride (25 μM) in the presence of brain mitochondria (70–90 μg of protein/ml). Each value is the mean ± SEM of 6–10 experiments with separate mitochondria. $^*P < 0.01$ compared with the control (none) value. Open circle, 4 weeks old rats; closed circle, 50 weeks old rats.

4 weeks old rats was significantly increased after addition of regucalcin (10^{-10} to 10^{-8} M) into the enzyme reaction mixture containing 25 μM calcium chloride. However, the enzyme activity in 50 weeks old rats was significantly increased with 10^{-8} M regucalcin addition. The effect of regucalcin in increasing Ca^{2+}-ATPase activity in rat brain mitochondria was weakened with aging. Meanwhile, Mg^{2+}-ATPase activity in the mitochondria of brain tissues obtained from 4 weeks old rats or 50 weeks old rats was not significantly changed by the addition of regucalcin (10^{-11} to 10^{-8} M) into the enzyme reaction mixture (data not shown).

Effects of Inhibitor on Regucalcin-Increased Mitochondrial Ca^{2+}-ATPase Activity

Ruthenium red or $LaCl_3$ is an inhibitor of mitochondrial uniporter activity [Nicholls and Akerman, 1982]. Whether the effect of regucalcin in increasing Ca^{2+}-ATPase activity in the mitochondria of brain tissues obtained from 4 weeks old rats is changed in the presence of ruthenium red or $LaCl_3$, was examined. Addition of ruthenium red (10^{-7} or 10^{-6} M) caused a significant decrease in Ca^{2+}-ATPase activity in the reaction mixture containing 25 μM calcium chloride (Fig. 3A). The effect of regucalcin (10^{-9} M) in increasing Ca^{2+}-ATPase activity was inhibited in the presence of ruthenium red (10^{-7} M; Fig. 3B).

Mitochondrial Ca^{2+}-ATPase activity was significantly decreased by the addition of $LaCl_3$ (10^{-7} or 10^{-6} M) in the enzyme reaction mixture (Fig. 4A). The effect of regucalcin (10^{-9} M) in increasing Ca^{2+}-ATPase activity was not seen in the presence of $LaCl_3$ (10^{-7} M; Fig. 4B).

Effect of Intracellular Signaling Factor on Regucalcin-Increased Mitochondrial Ca^{2+}-ATPase Activity

Whether the effect of regucalcin on Ca^{2+}-ATPase activity in the mitochondria of brain tissues obtained from 4 weeks old rats is mediated through calmodulin or DcAMP, which is related to intracellular signaling, was examined. Addition of calmodulin (1, 2.5, or 5 μg/ml of enzyme reaction mixture) into the reaction mixture containing 25 μM calcium chloride

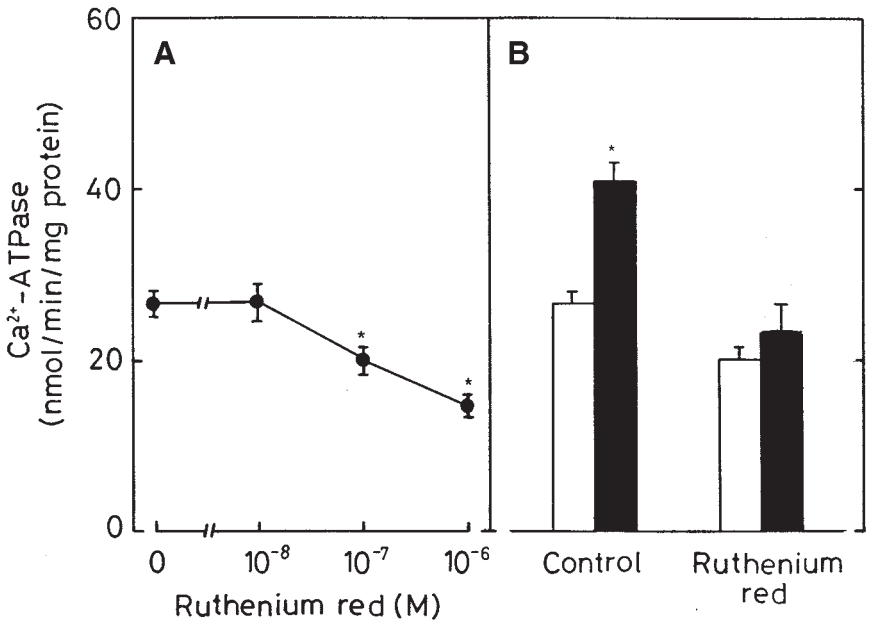

Fig. 3. Effect of ruthenium red on the regucalcin-increased Ca^{2+}-ATPase activity in rat brain mitochondria. Brain mitochondria was obtained from 4 weeks old rats. **A**: The enzyme reaction mixture contained either vehicle or ruthenium red (10^{-8} to 10^{-6} M) in the presence of mitochondria (70–90 μg/ml). **B**: The enzyme reaction mixture contained vehicle or regucalcin (10^{-9} M) with or without ruthenium red (10^{-7} M) in the presence of mitochondria (70–90 μg/ml). Each value is the mean ± SEM of 6 experiments with separate mitochondria. *$P < 0.01$ compared with the control (none) value. White bars, without regucalcin, black bars, with regucalcin.

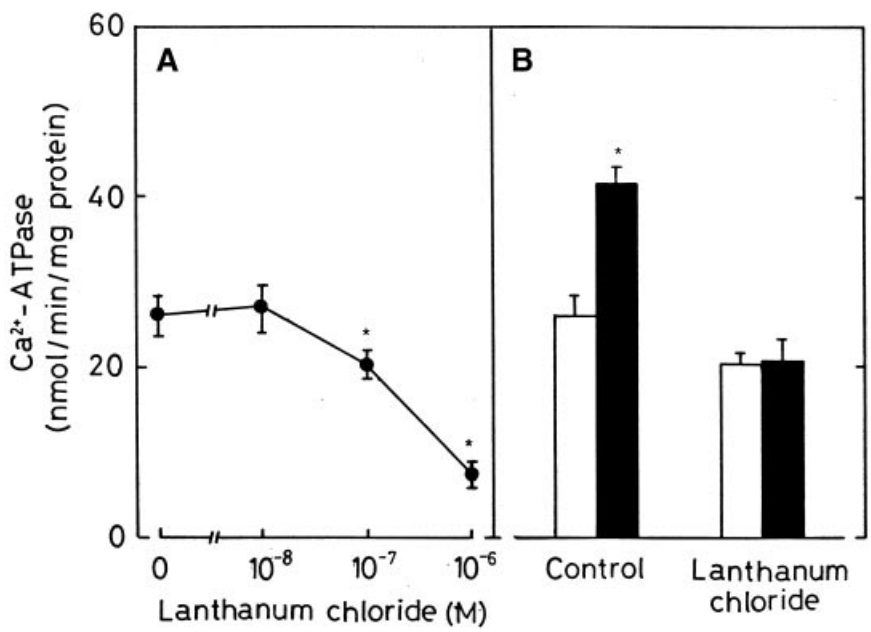

Fig. 4. Effect of lanthanum chloride on the regucalcin-increased Ca^{2+}-ATPase activity in rat brain mitochondria. Brain mitochondria was obtained from 4 weeks old rats. **A**: The enzyme reaction mixture contained either vehicle or lanthanum chloride (10^{-8} to 10^{-6} M) in the presence of mitochondria (60–90 μg/ml). **B**: The enzyme reaction mixture contained either vehicle or regucalcin (10^{-9} M) with or without lanthanum chloride (10^{-7} M) in the presence of mitochondria (68–95 μg/ml). Each value is the mean ± SEM of six experiments with separate mitochondria. *$P < 0.01$ compared with the control (none) value. White bars, without regucalcin, black bars, with regucalcin.

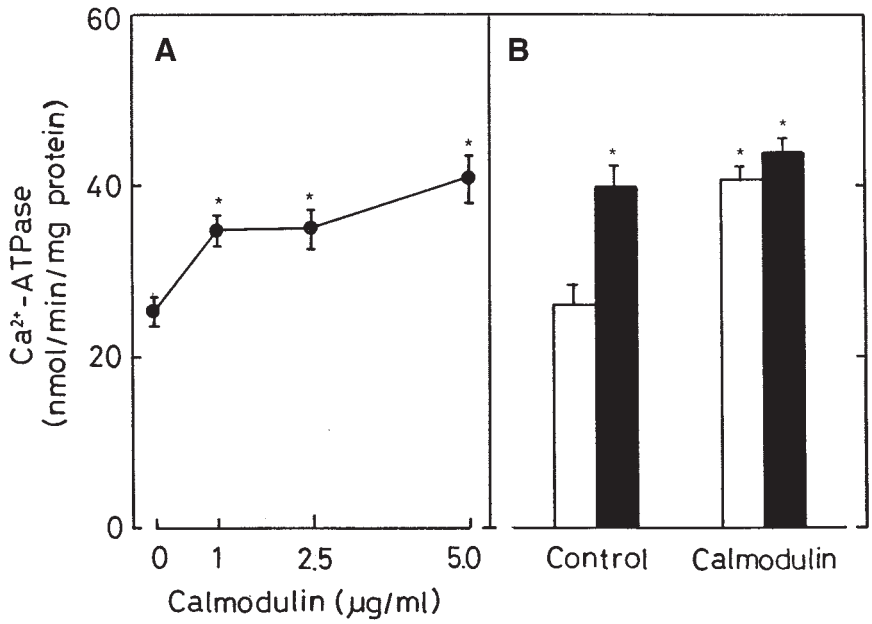

Fig. 5. Effect of calmodulin on the regucalcin-increased Ca^{2+}-ATPase activity in rat brain mitochondria. Brain mitochondria was obtained from 4 weeks old rats. **A**: The enzyme reaction mixture contained either vehicle or calmodulin (1, 2.5, or 5.0 μg/ml). **B**: The enzyme reaction mixture contained either vehicle or calmodulin (2.5 μg/ml) in the presence of mitochondria (70–103 μg/ml). B: Enzyme reaction mixture contained either vehicle or calmodulin (2.5 μg/ml) with or without regucalcin (10^{-9} M) in the presence of mitochondria (68–103 μg/ml). Each value is the mean ± SEM of six experiments with separate mitochondria. $^*P < 0.01$ compared with the control (none) value. White bars, without regucalcin, black bars, with regucalcin.

caused a significant increase in Ca^{2+}-ATPase activity (Fig. 5A). The effect of regucalcin (10^{-9} M) in increasing Ca^{2+}-ATPase activity was not significantly changed in the presence of calmodulin (2.5 μg/ml; Fig. 5B).

Addition of DcAMP (10^{-6} to 10^{-4} M) into the enzyme reaction mixture containing 25 μM calcium chloride did not have a significant effect on Ca^{2+}-ATPase activity (Fig. 6A). However, the effect of regucalcin in increasing Ca^{2+}-ATPase activity was not seen in the presence of DcAMP (10^{-4} M; Fig. 6B).

Change in Mitochondrial Ca^{2+}-ATPase Activity in Regucalcin Transgenic (RC TG) Rats

The change in Ca^{2+}-ATPase activity in the mitochondria of brain tissues obtained from 7 weeks old rats was examined. Regucalcin levels, which were determined using Western blot analysis, were significantly increased in the brain tissues (homogenate) or mitochondria obtained from RC TG rats as compared with those of normal (wild-type) rats (Fig. 7). Ca^{2+}-ATPase activity in the reaction mixture containing 25 μM calcium chloride was significantly increased in the mitochondria of RC TG rats (Fig. 8).

DISCUSSION

The neuronal cells have different ion transport system that contributes to the regulation of the low intracellular free Ca^{2+} concentration. Intracellular organelles that are capable of Ca^{2+} uptake such as mitochondria, endoplasmic reticulum, and other vesicular structures, may be involved in controlling cytoplasmic Ca^{2+} levels by sequestering and releasing Ca^{2+} [Heizmann and Hunziker, 1991]. Various buffering and transport systems like the Na^+/Ca^{2+} exchanger, Ca^{2+} channel, Ca^{2+}-ATPase, Ca^{2+}-binding proteins and intracellular Ca^{2+} uptake systems have been implicated in intracellular Ca^{2+} levels [Trotta and De Meis, 1978; Schellenberg and Swanson, 1981; MacDermott and Dale, 1986; Treves et al., 1990; Dahan et al., 1991; Salvador and Mata, 1996]. Regulation of Ca^{2+} homeostasis in neuronal cells has been demonstrated to be altered by aging [Heizmann and Hunziker, 1991; Magnoni et al., 1991; Hanahisa and Yamaguchi, 1998].

Aging has been shown to increase brain microsomal Ca^{2+}-ATPase activity in inducing an increase in intracellular calcium content [Hanahisa and Yamaguchi, 1998], and this increase is suppressed by regucalcin that has

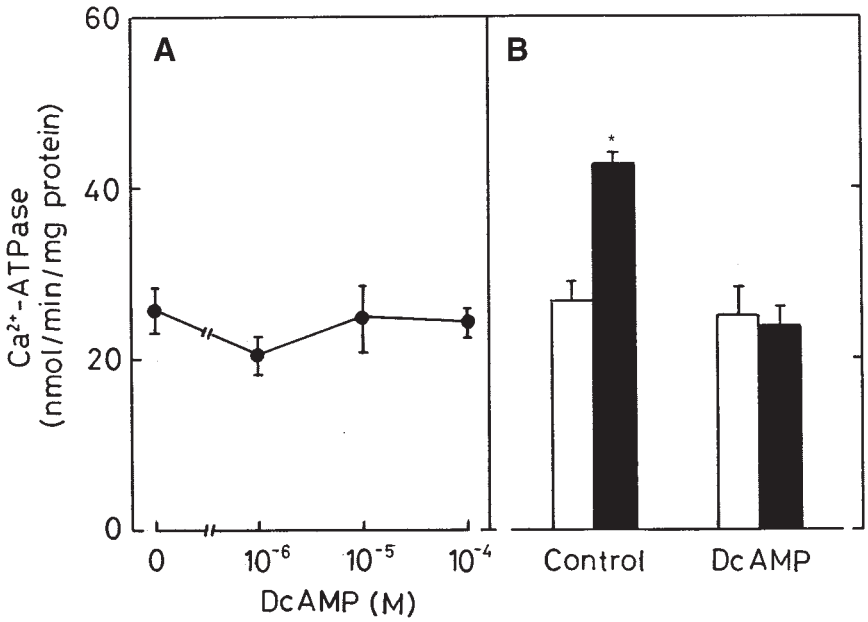

Fig. 6. Effect of dibutyryl cyclic AMP (DcAMP) on the regucalcin-increased Ca^{2+}-ATPase activity in rat brain mitochondria. Brain mitochondria was obtained from 4 weeks old rats. **A**: The enzyme reaction mixture contained either vehicle or DcAMP (10^{-6} to 10^{-4} M) in the presence of mitochondria (68–103 μg/ml). **B**: The enzyme reaction mixture contained either vehicle or DcAMP (10^{-4} M) with or without regucalcin (10^{-9} M) in the presence of mitochondria (68–103 μg/ml). Each value is the mean ± SEM of six experiments with separate mitochondria. $^*P < 0.01$ compared with the value of DcAMP alone with regucalcin. White bars, without regucalcin, Black bars, with regucalcin.

an inhibitory effect on microsomal Ca^{2+}-ATPase activity in rat brain tissues [Yamaguchi et al., 1999]. In addition, the inhibitory effect of regucalcin on brain microsomal Ca^{2+}-ATPase activities was atteneuated with aging. From these observations, it is assumed that regucalcin has a role in preventing an increase in brain calcium content with aging.

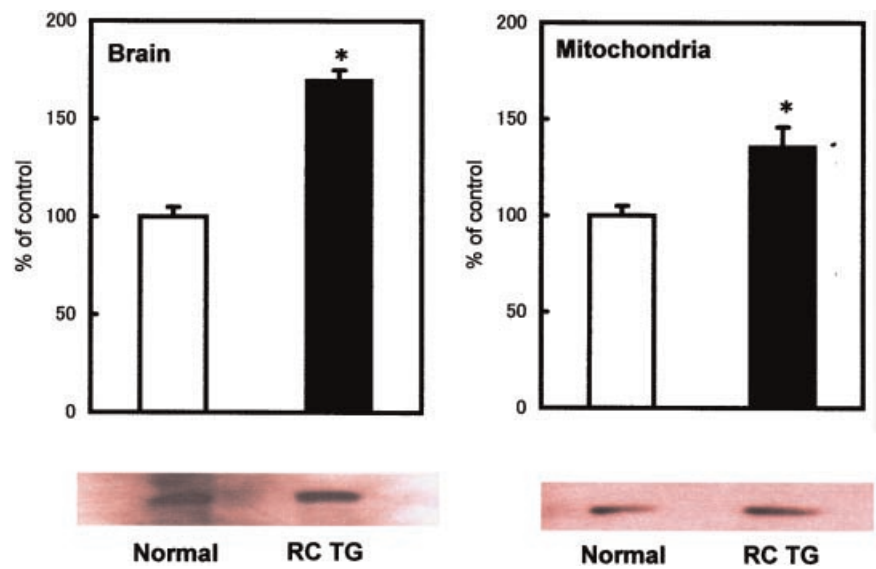

Fig. 7. Change in regucalcin in the brain mitochondria of regucalcin transgenic (RC TG) rats using Western blot analysis. The brain tissues (homogenate) or mitochondria were obtained from the brain tissues of normal rats or RC TG rats of 7 weeks old. Proteins (40 μg) of the homogenate or mitochondria prepared from the brain tissues of normal or RC TG rats were analyzed using Western blot analysis. The figure shows one of four experiments with separate rats. $^*P < 0.01$ compared with the value obtained from normal rats. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

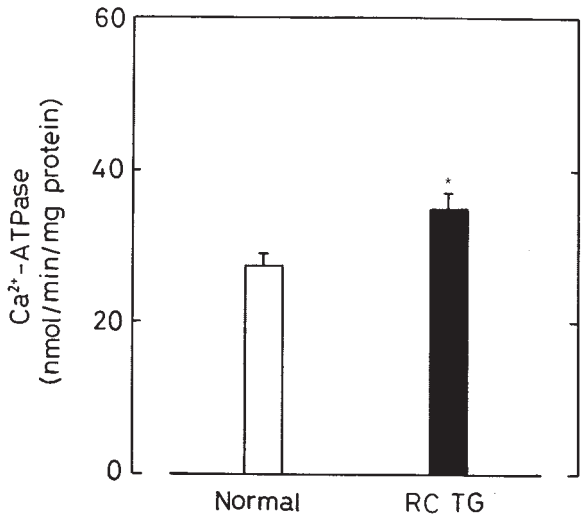

Fig. 8. Change in Ca^{2+}-ATPase activity in the mitochondria of regucalcin transgenic (RC TG) rats. The enzyme activity was measured in the mixture containing the mitochondria (60–90 μg of protein/ml) obtained from normal or RC TG rats in the presence of calcium chloride (25 μM). Each value is the mean ± SEM of four rats. $*P < 0.01$ compared with the value obtained from normal rats.

This study, furthermore, found that regucalcin significantly increased Ca^{2+}-ATPase activity in the mitochondria of rat brain tissues, and that the effect was weakened in brain mitochondria of aged rats. The concentration of regucalcin in rat brain tissues is found to be about 5×10^{-9} M, and it is reduced by increasing age [Yamaguchi et al., 1999]. The effect of regucalcin in increasing mitochondrial Ca^{2+}-ATPase activity in rat brain tissues was seen in the range of 10^{-9} to 10^{-8} M regucalcin. Presumably, regucalcin plays a physiologic and pathophysiologic roles in the regulation of the intracellular Ca^{2+} concentration in the brain tissues.

A Ca^{2+} carrier of the mitochondria has been reported to be a Ca^{2+}-binding glycoprotein [Panfili et al., 1980], but the energy of respiration or of ATP hydrolysis is utilized to generate a membrane potential (and a proton gradient) that is responsible for driving Ca^{2+} electrophoretically [Brand and Lehninger, 1975; Nicholls, 1978]. The effect of regucalcin in increasing mitochondrial Ca^{2+}-ATPase activity in rat brain tissues 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 brain mitochondria.

Calmodulin has been shown to be present in mitochondria [Gazzotti et al., 1984; Hatase et al., 1985]. We found that calmodulin increased Ca^{2+}-ATPase activity in rat brain mitochondria, suggesting that calmodulin can stimulate Ca^{2+} uptake by the mitochondria. The effect of regucalcin on the enzyme activity, however, was not further enhanced in the presence of calmodulin. Regucalcin and calmodulin may regulate reciprocally Ca^{2+} uptake activity in the mitochondria of brain tissues.

Whether DcAMP has a regulatory effect on brain mitochondrial Ca^{2+}-ATPase activity is not known in our knowledge. DcAMP did not have an effect on brain mitochondrial Ca^{2+}-ATPase activity, although DcAMP has been reported to increase Ca^{2+}-ATPase activity in rat heart microsomes [Yamaguchi and Nakajima, 2002]. The effect of regucalcin in increasing mitochondrial Ca^{2+}-ATPase activity in rat brain tissues was not seen in the presence of DcAMP. This result suggests that DcAMP can regulate the effect of regucalcin on mitochondrial Ca^{2+}-ATPase. The effect of DcAMP on regucalcin-stimulated Ca^{2+}-ATPase activity may be independent on protein kinase A, since the enzyme is not present in the brain mitochondria. It is possible that DcAMP inhibits the binding of regucalcin on the mitochondrial membranes. However, this remains to be elucidated. DcAMP is a derivative compound of cyclic AMP that is intracellular signaling factor. It is speculated that the effect of regucalcin in increasing mitochondrial Ca^{2+}-ATPase activity is regulated by cyclic AMP which generates following hormonal stimulation in brain tissues.

RC TG rats are generated to determine the role of endogenous regucalcin in vivo [Yamaguchi et al., 2002]. Regucalcin levels were increased in the mitochondria of brain tissues of RC TG rats, and the mitochondrial Ca^{2+}-ATPase activity was significantly elevated in the brain tissues of RC TG rats. These findings demonstrate that endogenous regucalcin can increase Ca^{2+}-ATPase activity in the mitochondria of rat brain tissues. Regucalcin may play a role as an activator of Ca^{2+}-ATPase in rat brain mitochondria.

In conclusion, it has been demonstrated that regucalcin increases Ca^{2+}-ATPase activity in the mitochondria of rat brain tissues, and that the enzyme activity is also elevated in RC TG rats.

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