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Inhibitory effect of calcium-binding protein regucalcin on Ca^{2+}-activated DNA fragmentation in rat liver nuclei

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Incubation of isolated rat liver nuclei with ATP, NAD^+, and micromolar Ca^{2+} concentrations of various metal ions resulted in extensive DNA hydrolysis. Half-maximal activity occurred with 1.0 μM Ca^{2+} added, and saturation of the process was observed with 10 μM Ca^{2+}. The Ca^{2+} (10 μM)-activated DNA fragmentation was inhibited by the presence of Ca^{2+}-binding protein regucalcin isolated from rat liver cytosol. The inhibitory effect of regucalcin was complete at 0.5 μM. At 25 μM Ca^{2+} added, such an effect of regucalcin (1.0 μM) was not seen. Regucalcin also inhibited Ca^{2+}-activated DNA fragmentation in the presence of calmodulin (10 and 20 μg). The results show that regucalcin can inhibit the Ca^{2+}-activated DNA fragmentation due to binding the metal, suggesting a role in regulation of liver nuclear functions.

Calcium; Regucalcin; DNA-fragmentation; Rat liver nucleus

1. INTRODUCTION

Ca^{2+} plays an important role in the regulation of many cell functions [1]. The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations [2,3]. Liver metabolism is regulated by the increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation. Recent accumulating evidence suggests that Ca^{2+} plays a role in liver nuclear function [4–9]. Calmodulin, a calcium-binding protein which can amplify Ca^{2+} effect [10], exists in rat liver nuclei [4]. The existence of an ATP-stimulated Ca^{2+} sequestration system in rat liver nuclei that requires calmodulin and generates a net increase in nuclear matrix free Ca^{2+} concentration has been reported [5]. Calmodulin stimulates DNA synthesis by liver cells [6], and the calmodulin effect may be mediated through α-adrenergic stimulation [7,8].

On the other hand, a novel calcium-binding protein (regucalcin), which differs from calmodulin [10] and other calcium-binding proteins (caligulin [11], calregulin [12] and calreticulin [13]) is distributed in rat liver cytosol [14–16]. Regucalcin may play a cell physiological role different from those of other calcium-binding proteins in the regulation of liver cell functions; regucalcin can reverse the effect of Ca^{2+} on many enzymes in liver cells [17–20]. Regucalcin may play a role as a regulatory protein for Ca^{2+} effects in liver cells.

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More recently, it has been reported that isolated rat liver nuclei contain a DNA endonuclease activity dependent upon Ca^{2+} in the submicromolar range, and that Ca^{2+} results in extensive DNA hydrolysis [21]. Therefore, the present investigation was undertaken to clarify whether regucalcin has an effect on Ca^{2+}-activated DNA fragmentation in isolated rat liver nuclei. It was found that regucalcin inhibits Ca^{2+}-activated DNA fragmentation in the nuclei.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Adenosine 5'-triphosphate (ATP), nicotinamide adenine dinucleotide (NAD^+), ethyleneglycol-bis-(aminoethyl ether)*N,N'*-tetraacetic aid (EGTA), and calmodulin (52 000 units/mg protein from bovine brain) were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). $CaCl_2 \cdot 2H_2O$ and other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). The reagents were dissolved in distilled water and then passed through ion-exchange resin to remove metal ions.

2.2. *Isolation of regucalcin*

Male Wistar rats, weighing 100–120 g, purchased from the Japan Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca, and 1.1% P, and distilled water, freely. After one week on this diet animals were killed by bleeding. 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 in Tris-HCl buffer (pH 7.4) and the homogenate was spun at 5500 × *g* in a refrigerated centrifuge for 10 min and the supernatant was spun at 105 000 × *g* for 60 min. Regucalcin in the 105 000 × *g* supernatant (cytosol) 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 [14]. Protein concentration was determined by the method of Lowry et al. [22] using albumin as a standard.

2.3. *Isolation of nuclei*

Liver nuclei were isolated by the procedure of Jones et al. [21] with a minor modification. Rats were killed by cardiac puncture, and the liver was perfused with approximately 10 ml of ice-cold TKM solution (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$) to remove blood. Livers were then removed, cut into small pieces, and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in 40 ml of the same solution containing 0.25 M sucrose. The homogenate was filtered through three layers of cheesecloth. The nuclei were pelleted by centrifugation at 700 × *g* for 10 min. The pellets were homogenized (five strokes) in 40 ml of the same solution and centrifuged again at 700 × *g* for 10 min. The pellet was resuspended in 24 ml of the same solution by homogenization (five strokes), and 6 ml were added to each of four tubes containing 12 ml of TKM containing 2.3 M sucrose solution. The tubes were gently mixed, and a 6-ml cushion (TKM containing 2.3 M sucrose) was carefully layered on the bottom of each tube. The tubes were centrifuged at 37 000 × *g* for 30 min. The upper layer and the sucrose cushion were removed with an aspirator. The resulting pellet of highly purified nuclei was resuspended in the incubation medium (125 mM KCl, 2 mM potassium phosphate, 25 mM Hepes, 4 mM $MgCl_2$, pH 7.0) by hand homogenization. Assay of marker enzymes (glucose-6-phosphatase, 5'-nucleotidase, succinate dehydrogenase) showed that there was less than about 5% contamination by microsomes, plasma membranes, or mitochondria.

2.4. *Assay of DNA fragmentation*

Nuclei from one liver were suspended in 10 ml of incubation medium and divided into the appropriate number of flasks with additions as needed. The incubation mixture to assay in vitro DNA fragmentation contained, in a total of 2.4 ml, nuclei (1.0-1.5 mg of DNA), 1 mM ATP (adjusted to pH 7.0 with KOH), 1 mM NAD^+ and either 2 mM EGTA or the indicated concentration of $CaCl_2$, and unless otherwise indicated, regucalcin (0.25-2.0 μM) and/or calmodulin (10 and 20 μg), as described by Jones et al. [21]. After incubation at 37°C for 10 min, 2-ml aliquots were removed and added to 3 ml of ice-cold lysis medium (5 mM Tris-HCl, 20 mM EDTA, 0.01% (w/v) Triton X-100, pH 8.0). After 10 min, samples were centrifuged for 20 min at 27 000 × *g* to separate the intact chromatin (pellet) from the fragmented DNA (supernatant) [23]. The supernatants were decanted and saved; the pellets were resuspended in 5 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Pellet and supernatant fractions were assayed for DNA content with the diphenylamine reaction [24]. DNA fragmentation was expressed as percentage of supernatant DNA for total DNA (supernatant plus pellet DNA) which resisted centrifugation at 27 000 × *g*.

2.5. *Statistical methods*

The significance of differences between values was estimated by using Student's *t*-test. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference.

3. RESULTS

The effect of various metals on in vitro DNA fragmentation in isolated rat liver nuclei is shown in Fig. 1. When liver nuclei were added into the assay mixture containing various metals (10 μM), the fragmentation of DNA in the nuclei was significantly increased by the presence of $CaCl_2$. Meanwhile, $ZnCl_2$, $AlCl_3$, $CdCl_2$, $MnCl_2$, $CoCl_2$ and $NiCl_2$ did not have an appreciable effect on DNA fragmentation in the nuclei. Thus, of the various metal ions, Ca^{2+} could uniquely activate DNA fragmentation in rat liver nuclei.

The effect of increasing concentrations of added Ca^{2+} on the fragmentation of DNA in rat liver nuclei is shown in Fig. 2. Addition of 0.1 μM Ca^{2+} had no effect on DNA fragmentation (data not shown). Ca^{2+} up to 1.0 μM Ca^{2+} caused a significant increase in DNA fragmentation, and the increase was saturated at 10 μM Ca^{2+}.

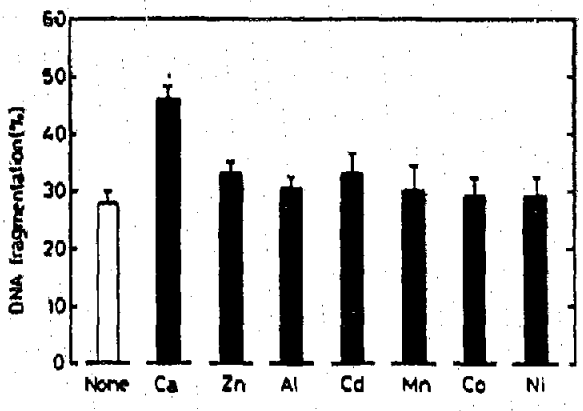

Fig. 1. Effect of various metals on DNA fragmentation in rat liver nuclei. The nuclei were incubated for 10 min in the reaction mixture containing either 2 mM EGTA or the indicated metal ion (10 μM). Each bar is the mean of values obtained from five rats per group. The vertical lines represent the SE. $^*P<0.01$, as compared with the value of control (none).

The effect of regucalcin, a calcium-binding protein, on the fragmentation of DNA in rat liver nuclei is shown in Fig. 3. An appreciable effect of regucalcin on Ca^{2+} (10 μM)-activated DNA fragmentation was not seen at 0.25 μM. However, the Ca^{2+}-activated DNA fragmentation was significantly inhibited by the presence of 0.5 μM regucalcin. With the greater concentrations (1.0 and 2.0 μM), the effect was saturated. Meanwhile, regucalcin (1.0 μM) did not have an effect

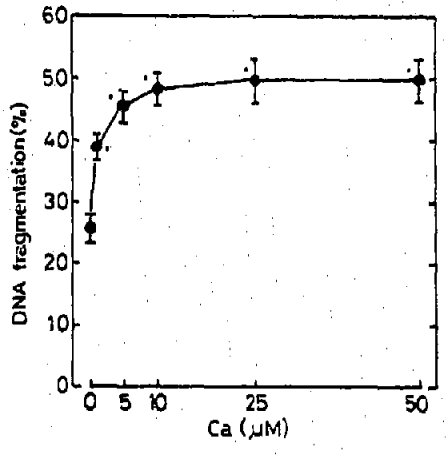

Fig. 2. Effect of increasing concentrations of Ca^{2+} on DNA fragmentation in rat liver nuclei. The nuclei were incubated for 10 min in the reaction mixture containing either 2 mM EGTA or $CaCl_2$ in the range of 1.0–5.0 μM as a final concentration added. Each point is the mean of values obtained from five rats per group. The vertical lines represent the SE. $^*P<0.01$, as compared with the value without Ca^{2+} addition.

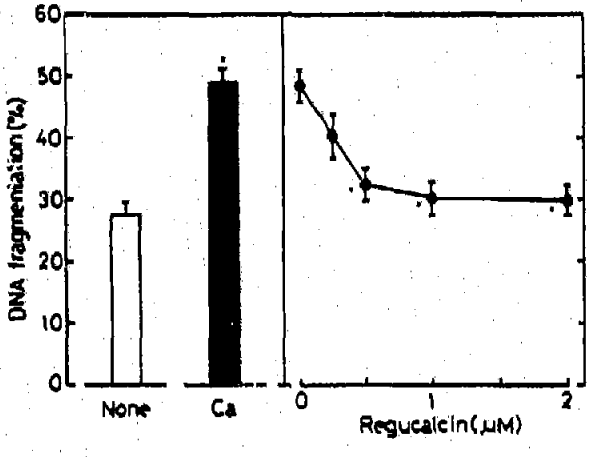

Fig. 3. Effect of increasing concentrations of regucalcin on DNA fragmentation in rat liver nuclei. The reaction mixture was incubated for 10 min in the presence of 10 μM $CaCl_2$ and regucalcin (0, 0.25, 0.50, 1.0 and 2.0 μM). Each bar or point is the mean of values obtained from five rats per group. The vertical lines represent the SE. *$P < 0.01$, as compared with the control value. (□) none; (■) 10 μM $CaCl_2$ addition.

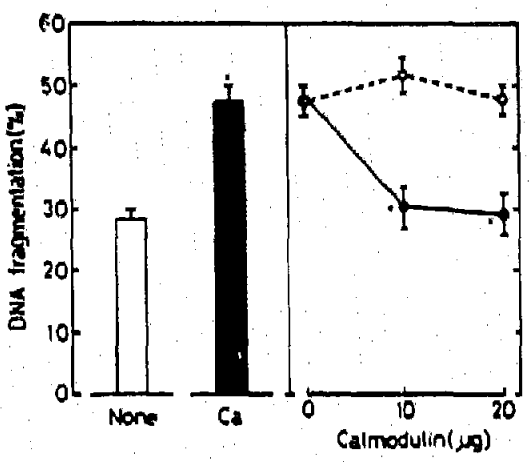

Fig. 5. Effect of regucalcin on DNA fragmentation with calmodulin in rat liver nuclei. The reaction mixture was incubated for 10 min in the presence of 10 μM $CaCl_2$, calmodulin (0, 10 and 20 μg) and 1.0 μM regucalcin. Each bar or point is the mean of values obtained from five rats per group. The vertical lines represent the SE. *$P < 0.01$, as compared with the control value. (○) without regucalcin; (●) with regucalcin.

on DNA fragmentation in the absence of Ca^{2+} (with 2 mM EGTA).

When the concentration of Ca^{2+} added in the reaction mixture with liver nuclei was varied in the range of 5.0–50 μM with 1.0 μM regucalcin, the fragmentation of DNA in the nuclei increased at a higher concentration (25 and 50 μM) of the metal (Fig. 4). In the presence of 5.0 and 10 μM Ca^{2+} added, regucalcin (1.0 μM) completely prevented an increase in the nuclear DNA fragmentation caused by the metal. Thus, the inhibitory effect of regucalcin (1.0 μM) was seen at a comparatively lower concentration (less than 10 μM) of Ca^{2+} added.

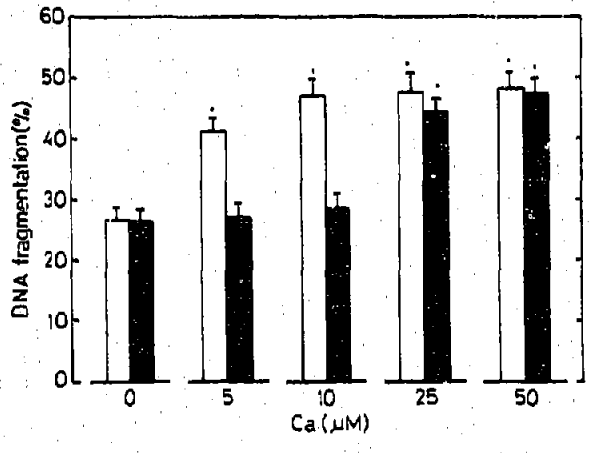

Fig. 4. Effect of regucalcin on DNA fragmentation with variation of Ca^{2+} concentrations in rat liver nuclei. The reaction mixture was incubated for 10 min in the presence of $CaCl_2$ (0, 5, 10, 25 and 50 μM) and regucalcin (1.0 μM). Each bar is the mean of values obtained from five rats per group. The vertical lines represent the SE. *$P < 0.01$, as compared with the value of control (none). (□) none; (■) 1.0 μM regucalcin addition.

In the presence of 10 μM Ca^{2+} added, DNA fragmentation in liver nuclei was not significantly enhanced by the presence of calmodulin (10 and 20 μg) (Fig. 5). The inhibitory effect of regucalcin (1.0 μM) on Ca^{2+} (10 μM)-activated DNA fragmentation was seen in the presence of calmodulin (10 and 20 μg).

4. DISCUSSION

A novel calcium-binding protein regucalcin, which differs from calmodulin and other calcium-binding proteins [11–13], is distributed in the hepatic cytosol of rats [15,16]. The molecular weight of regucalcin was estimated to be 28 800 [15]. The Ca^{2+} binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis, and there appears to be 6–7 high affinity binding sites for Ca^{2+} per molecule of protein [15]. Amino acid analysis of regucalcin showed glycine and glutamic acid to be the predominant amino acid [15]. The isoelectric point of regucalcin is 5.20; from the circular dichroism spectrum, the apparent α-helical content of regucalcin in Ca^{2+}-free buffer is estimated to be 34% [16], and this value is decreased by 1.0 mM Ca^{2+} addition [16]. Regucalcin may play a cell physiological role different from those of other calcium-binding proteins in the regucalcin of liver cell functions. This novel protein can reverse the effect of Ca^{2+} on many enzymes in hepatic cytosol [17,19,25].

More recent investigations suggest that Ca^{2+} plays an important role in the regulation of liver nuclear functions [4–9]. Previous work has shown that rapid autodigestion of DNA can occur when nuclei isolated

from a variety of different tissues, including rat liver, are incubated in a buffer containing Ca^{2+} and Mg^{2+} [26,27]. The Ca^{2+} dependence of this endogenous DNA fragmentation process in more detail has been examined in a recent study [21]; it shows that isolated rat liver nuclei contain a DNA endonuclease activity dependent upon Ca^{2+} in the submicromolar range when the nuclei are reconstituted with NAD^+ and ATP [21]. This endogenous endonuclease activity may be responsible for the DNA fragmentation occurring during programmed cell death and certain types of toxic cell killing.

In the present investigation, it has been demonstrated that Ca^{2+}, of various metals, can uniquely stimulate in vitro DNA fragmentation in isolated rat liver nuclei. A significant increase was seen by addition of 1.0 μM Ca^{2+}. This result coincides with previous other work [21]. The presence of regucalcin (0.5–2.0 μM) completely inhibited the activation of liver nuclear DNA fragmentation by addition of 10 μM Ca^{2+}. This inhibition was not seen by addition of Ca^{2+} at 25 and 50 μM. Thus, regucalcin had an effect on DNA fragmentation with a comparatively lower concentration of Ca^{2+} (5.0 and 10 μM) used. The regucalcin effect on DNA fragmentation may be based on binding Ca^{2+}, since there appear to be 6–7 high affinity binding sites for Ca^{2+} per molecule of regucalcin [15].

DNA fragmentation in rat liver nuclei may be stimulated by Ca^{2+}-calmodulin [21], and calmodulin exists in liver nuclei [4]. In the present study, addition of calmodulin (10 and 20 μg) did not cause an appreciable increase of Ca^{2+} (10 μM)-activated DNA fragmentation in liver nuclei. The endogenous calmodulin may have a sufficient effect to enhance Ca^{2+}-activated DNA fragmentation in liver nuclei. However, the presence of regucalcin caused an inhibition of Ca^{2+}-activated DNA fragmentation due to Ca^{2+} addition. Such inhibition was also seen in the presence of exogenous calmodulin. Presumably, regucalcin can regulate Ca^{2+}/calmodulin-dependent DNA fragmentation in liver nuclei. Radioiodinated regucalcin can bind to the nuclei isolated from rat liver in the absence or presence of 1.0 mM Ca^{2+} [18].

Several recent studies have indicated that Ca^{2+} plays an important role in regulation of nuclear functions [4–9,21]. Also, it has been found that a sustained increase in cytosolic Ca^{2+} level precedes the activation of DNA fragmentation that is characteristic of programmed cell death (apoptosis) and in certain forms of chemically induced cell killing [28,29]. The present finding, that regucalcin prevents the activation of DNA fragmentation by Ca^{2+}, suggests a cell physiological role in liver cells. Regucalcin may have a role as regulatory protein for Ca^{2+} action.

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