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Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver

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The distribution and expression of mRNA encoding the Ca^{2+}-binding protein regucalcin in rats were investigated by Northern blot analyses. Liver regucalcin cDNA (0.6 kb) was used as a probe. The analyses of total RNAs extracted from various tissues of rat indicated that regucalcin mRNA was mainly present in liver but only slightly in kidney with a size of 1.8 kb. The expression level decreased with increasing age (3, 10 and 25 weeks). A single intraperitoneal administration of calcium chloride (15 mg Ca/100 g body weight) induced a remarkable increase in regucalcin mRNA in liver; the level was about 200% of control at 30 min after the administration. Subsequently, the expression level began to decrease with time and was about 40% of control level at 120 min after the administration. The increase in regucalcin mRNA levels at 30 min after calcium administration was dose-dependent. These observations show that the expression of regucalcin mRNA is specific in liver of various tissues, and that it is regulated by Ca^{2+} administration. Regucalcin may have a role as regulatory protein for calcium homeostasis in liver cells.

Regucalcin; Calcium; Gene expression; Northern blot analysis; Rat liver

1. INTRODUCTION

Ca^{2+} plays a role as an important second messenger signal in a variety of pathways to produce a Ca^{2+}-mediated physiological response in many cells. The Ca^{2+} signal is transmitted to an intracellular response partly via a family of calcium-binding proteins [1]. Recently, it has been found that a novel calcium-binding protein, regucalcin, is distributed in the hepatic cytosol of rats [2]. The mol. wt. of regucalcin was estimated to be 28,800, and the Ca^{2+} binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis [3]. Regucalcin has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells [4–8]. The physiological role of regucalcin may differ from that of calmodulin in which can amplify the Ca^{2+} effect on liver metabolism [9].

On the other hand, tissue distribution and expression of the regucalcin gene has not been reported. Therefore, the present investigation was undertaken to clarify the tissue distribution and regulation of regucalcin-messenger ribonucleic acid (mRNA) by Northern blot analyses using hepatic regucalcin complementary deoxyribonucleic acid (cDNA) as a probe. It was found that regucalcin mRNA is mainly distributed in liver, and that the expression is increased by the administration of calcium.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Deoxycytidine 5′-[α^{32}P] triphosphate ([^{32}P]dCTP; 110 Tbq/mmol) and nylon membrane (Hybond N$^+$) for Northern hybridization were obtained from Amersham (Buckinghamshire, UK). A human β-actin gene fragment (0.43 kb) as an internal standard was obtained from Wako Pure Chemical Co. (Osaka, Japan). Molecular size standards (0.24–9.5 kb RNA ladder) for electrophoresis of RNA were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Calcium chloride and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Wako Pure Chemical Co. Any water and solutions used for RNA preparation were treated with chemical diethylpyrocarbonate (DEPC, Sigma) to inhibit RNase activity.

2.2. *Animals and tissues*

Wistar male rats of different ages, purchased from Japan SLC Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid, Oriental Yeast Co., Tokyo) containing 57.5% carbohydrate, 1.1% calcium and 1.1% phosphorus and distilled water, freely.

The following tissues were dissected from rats (3-week-old) to analyze the tissue distribution of regucalcin mRNA; cerebrum, cerebellum, heart, lung, liver, spleen, kidney, skeletal muscle and smooth muscle (diaphragm). Also, 10- and 25-week old rats were used to analyse the expression of regucalcin mRNA during aging.

2.3. *Calcium administration*

Calcium chloride was dissolved in sterile distilled water at concentrations of 5, 15 and 30 mg Ca per ml. These solutions were intraperitoneally administered to rats. At 30, 60 and 120 min after calcium administration, the rats were sacrificed by bleeding. The livers were perfused with ice-cold 0.25 M sucrose solution and immediately removed and frozen at −80°C. Control animals received vehicle solution.

2.4. *Isolation of RNA*

Total RNAs were prepared as described [10]. Liver and other tissues were quickly removed, rinsed with ice-cold 0.25 M sucrose solution,

and homogenized in buffer solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol and 2 M sodium acetate. Total RNAs were extracted by vigorous shaking in a mixture of phenol, chloroform 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 50 μl of DEPC-treated 0.5% sodium dodecyl sulfate (SDS).

2.5. *Northern blotting*

Ten micrograms of total RNAs extracted from each tissue were electrophoresed in 1.2% agarose denaturing gels containing 2.2 M formaldehyde in MOPS buffer (pH 7, containing 20 mM 3-*N*-morpholino-propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA), applying 3 V/cm for 3 h [11]. The electrophoresed gels were transferred to nylon membrane by blotting [11]. Part of regucalcin cDNA (a 0.6 kb, *KpnI–PstI* insert) was labeled with [^{32}P]dCTP by random primers with the DNA polymerase Klenow fragment [12]. This radioactive probe was used for hybridization detection of RNAs on blots. The membranes were prehybridized, and hybridized in buffer solution containing 50% formamide, 5 × SSPE (1 × SSPE; 1.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5 × Denhardt's reagent; (1 × Denhardt's reagent; 0.02% (w/v) each of bovine serum albumin, Ficoll and polyvinylpyrrolidine) and 0.5% SDS with ^{32}P-labeled regucalcin cDNA in a sealed plastic bag at 42°C for 16 h. After hybridization the membranes were washed as follows: 2 × SSPE and 0.1% SDS at 42°C (twice for 15 min), followed by 0.1 × SSPE and 0.1% SDS at room temperature (twice for 15 min), and then the membranes were exposed to X-ray film for 12 h.

The quantity and integrity of mRNA were monitored by rehybridizing with a radioactive cDNA probe from the human β-actin gene fragment under identical conditions. No noticeable change in the level of RNA hybridized with the β-actin probe was observed throughout the present experiments (data not shown). The size of the hybridizing RNA was determined by running the standard RNA molecules of known size in parallel. The density of the autoradiographic data was quantified by densitometer scanning (Dual-wavelength Flying-spot Scanner, CS-9000, Shimadzu Co., Japan).

3. RESULTS

The tissue distribution of regucalcin mRNA in rats is shown in Fig. 1. In all four types of preparation studied, Northern blot analysis using the regucalcin cDNA probe showed that regucalcin mRNA was expressed in the liver and kidney, while it was not detected in the cerebrum, cerebellum, heart, lung, spleen, skeletal muscle and smoot muscle (diaphragm). The liver had high levels of regucalcin mRNA expression, while the kidney contained comparatively low levels. In both tissues, two distinct mRNA isoforms of 1.8 kb and 1.6 kb were seen. The major regucalcin mRNA band was 1.8 kb.

The alteration of regucalcin mRNA in rat liver with increasing age is shown in Fig. 2. With increasing age, regucalcin mRNA levels decrease. Regucalcin mRNA levels at 25 weeks were about 50% of that of 3-week-old rats. Thus, hepatic regucalcin mRNA levels were reduced with increasing age.

The effect of calcium administration on regucalcin mRNA in the liver of rats is shown in Fig. 3. A solution of calcium chloride (15 mg Ca/100 g body weight) was intraperitoneally administered to rats, and the animals

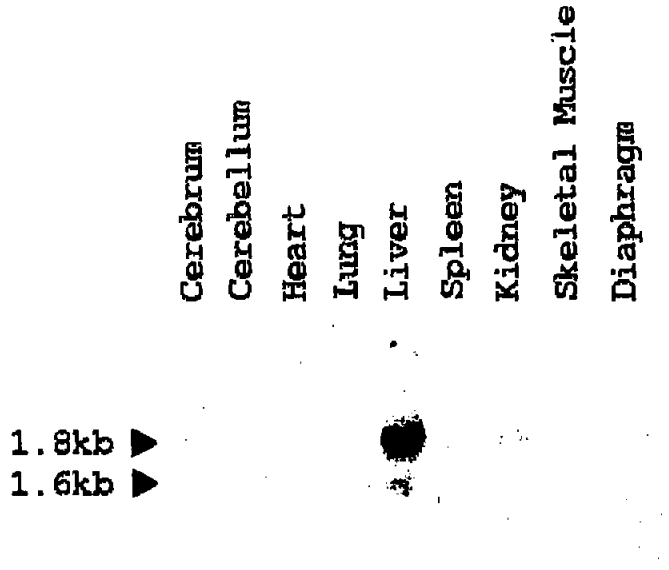

Fig. 1. Tissue distribution of regucalcin mRNA levels in rats. Total RNAs (10 μg) isolated from various tissues in rats were subjected to Northern blot analysis. The pattern of hybridization obtained with the rat liver regucalcin cDNA is shown. The arrowheads indicate hybridizing bands corresponding to mRNA encoding regucalcin. The result shows one of four experiments with separate rats.

were sacrificed at 30, 60 and 120 min after the administration. At 30 min after calcium administration, liver regucalcin mRNA was markedly increased; the level was about 200% of the control level. Subsequently, the mRNA level began to decrease, and it reduced to about 40% of the control level at 120 min after the calcium administration. Now, the extracted total RNA content was not changed by calcium administration; the RNA content was in the range from 0.9 to 1.1 μg RNA per mg wet liver tissue.

The effect of increasing doses of calcium (5, 15 and 30 mg/100 g) on regucalcin mRNA in the liver of rats

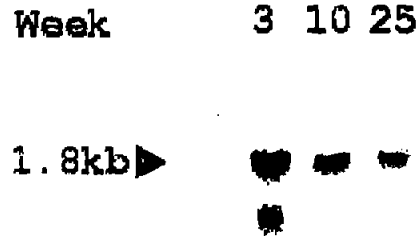

Fig. 2. The alteration of regucalcin mRNA level in the liver of rats with increasing age. In each lane 10 μg of total RNA extracted from the liver at different ages (3, 10 and 25 weeks) was subjected to Northern blot analysis. The pattern of hybridization obtained with the rat liver regucalcin cDNA is shown. The arrowhead indicates hybridizing bands corresponding to mRNA encoding the regucalcin. The result shows one of four experiments with separate rats.

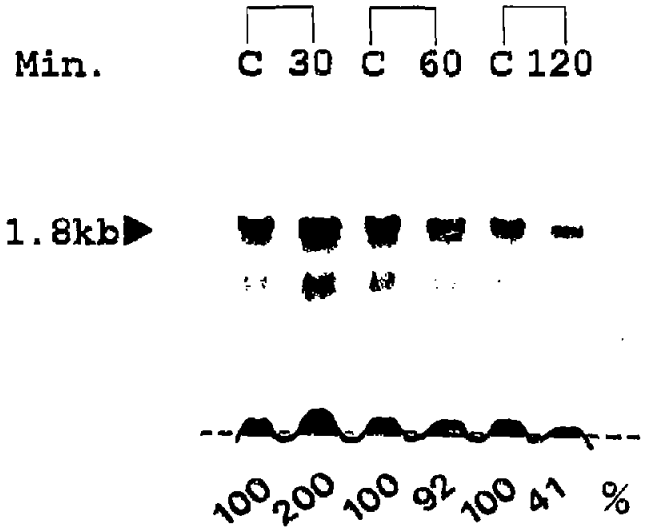

Fig. 3. Effect of calcium administration on regucalcin mRNA levels in the liver of rats. Animals received a single intraperitoneal administration of calcium (15 mg/100 g), and 30, 60 and 120 min later they were sacrificed by bleeding. Control animals (C) received an equivalent volume of the distilled water. Total RNA (10 μg) isolated from the liver were subjected to Northern blot analysis. The pattern of hybridization obtained with the rat liver regucalcin cDNA is shown. The arrowhead indicates hybridizing bands corresponding to mRNA encoding the regucalcin. The result shows one of four experiments with separate rats.

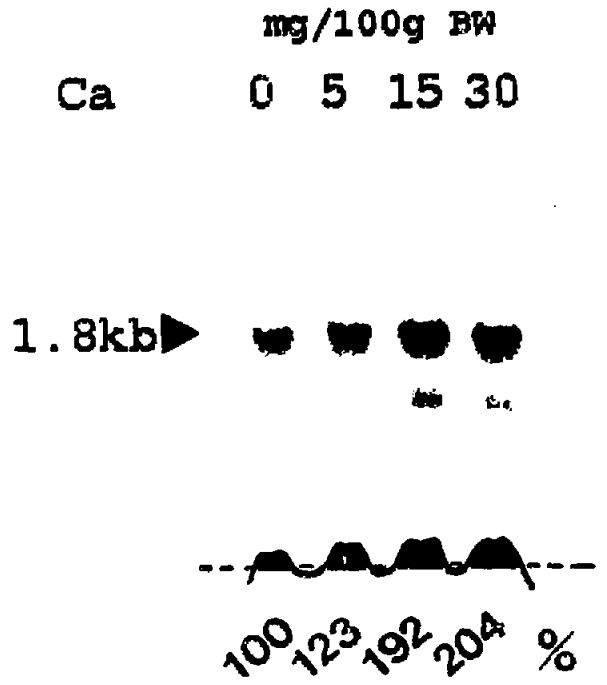

Fig. 4. The effect of increasing doses of calcium on regucalcin mRNA levels in the liver of rats. Animals received a single intraperitoneal administration of calcium (5, 15 and 30 mg/100 g), and 30 min later they were sacrificed by bleeding. Control animals (0) received an equivalent volume of the distilled water. Total RNAs (10 μg) isolated from the liver were subjected to Northern blot analysis. The pattern of hybridization obtained with the rat liver regucalcin cDNA is shown. The arrowhead indicates hybridizing bands corresponding to mRNA encoding the regucalcin. The result shows one of four experiments with separate rats.

is shown in Fig. 4. The liver was removed 30 min after a single intraperitoneal administration of calcium (5, 15 and 30 mg/100 g). The effect of calcium administration on regucalcin mRNA was dose-dependent. Higher doses (15 and 30 mg Ca/100 g) produced higher mRNA levels. The dose of 30 mg Ca/100 g produced a twofold increase over control levels.

4. DISCUSSION

A novel calcium-binding protein regucalcin, which differs from calmodulin and other calcium-binding proteins [13–15], is distributed in the hepatic cytosol of rats [2,3]. Regucalcin may play a physiological role in the cell different from that of calmodulin, which can amplify the Ca^{2+} effect on liver metabolism. Regucalcin has the reversible effect of Ca^{2+} on many enzymes in the hepatic cytosol [4–8]. More recently, it was reported that regucalcin can inhibit the activation of Ca^{2+}/calmodulin-dependent cyclic AMP phosphodiesterase [7], protein kinase C [5] and Ca^{2+}-activated DNA fragmentation [8] due to binding of Ca^{2+}.

The present investigation was undertaken to further clarify the role of regucalcin by determining the tissue distribution of regucalcin mRNA in rats. Regucalcin mRNA levels in rat tissues were examined by Northern blot analyses with a hepatic regucalcin cDNA probe. Regucalcin mRNA was mainly found in the liver and to a slight extent in the kidney, while it was not observed in brain, heart, lung, spleen and muscles. Most calcium-binding proteins are expressed in a number of tissues. Calcyclin mRNA is distributed over lung, kidney and uterus [16]. The tissue distribution of calcyphosine includes thyroid, salivary gland, kidney, lung and brain [17]. Thus, regucalcin is characteristic by its specific expression in liver. The present results reveal the existence of two distinct mRNA isoforms of 1.8 kb and 1.6 kb, although the 1.6 kb mRNA was only slightly present. These isoforms are thought to be generated by alternative splicing of common RNA precursor molecules; the 1.6 kb mRNA may be an intermediate molecule of the mature mRNA of regucalcin. Furthermore, with increasing age, regucalcin mRNA levels clearly decrease. This finding suggests that regucalcin synthesis in liver cells may deteriorate with advancing age.

The effects of Ca^{2+} on the expression of regucalcin mRNA in liver was also investigated. Calcium chloride was administered intraperitoneally to rats using a dose of 5–30 mg Ca/100 g. The administration of calcium results in a rapid increase in the expression levels of mRNA encoding regucalcin in the liver. The increase in regucalcin mRNA levels occurred during the thirty minutes after calcium administration. Thereafter, the levels

returned toward control levels and then decreased to less than about 50% of control levels. From these observations, two important implications are assumed. Firstly, the expression of regucalcin mRNA rapidly responds to calcium administration. Secondly, there may be a regulating system to suppress over-expression of the regucalcin gene in liver. It has been previously shown that a single intraperitoneal administration of calcium chloride (2.0 mg/100 g body weight) to rats produced a remarkable increase of calcium content in the liver [18]. Thus, the doses of calcium used were sufficient to increase the calcium content in the liver. Accordingly, liver calcium may play a role in the expression of regucalcin mRNA levels.

Several steps may be related to the regulation of regucalcin mRNA levels during the process of post-transcription; mRNA stability, translation and post-translational events. We have not established whether the change in the observed regucalcin mRNA levels is due to the transcription process or to mRNA stability. Additional studies designed to directly assess transcriptional rates and half-lives of regucalcin mRNA will be necessary.

In conclusion, it was demonstrated that regucalcin mRNA was specifically localized in the liver of rats, and that mRNA levels clearly increased during the early phase of calcium administration. The present findings suggest that regucalcin mRNA expression may be stimulated by increasing calcium in liver cells.

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