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Expression of calcium-binding protein regucalcin mRNA in rat liver is stimulated by calcitonin: the hormonal effect is mediated through calcium

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Abstract

The involvement of a hypocalcemic hormone calcitonin (CT) in the expression of hepatic Ca^{2+}-binding protein regucalcin mRNA was investigated. The change of regucalcin mRNA levels was analyzed by Northern blotting using liver regucalcin complementary DNA (0.9 kb). A single oral administration of calcium chloride (100 mg Ca/100 g body weight) to rats induced a remarkable increase in the serum calcium concentration and a corresponding elevation of the liver calcium content during 120 min after the administration. Thyroparathyroidectomy (TPTX) did not cause a significant increase in the liver calcium content after calcium administration. Hepatic regucalcin mRNA level was markedly elevated by calcium administration; the level was about 180% of controls at 60 min after the administration. This increase was completely abolished by TPTX. A single subcutaneous administration of CT (synthetic eel CT; 25 – 100 MRC mU/100 g) to TPTX rats received oral administration of calcium (100 mg/100 g) produced a remarkable increase in hepatic regucalcin mRNA levels; the level was about 280% of controls with the dose of 25 MRC mU CT/100 g. The present finding suggests that the expression of hepatic mRNA is stimulated by CT, and that the hormonal effect is mediated through Ca^{2+} in rat liver. (Mol Cell Biochem **136:** 43–48, 1994)

Key words: regucalcin, calcium-binding protein, gene expression, rat liver

Introduction

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. Liver metabolism is regulated by an increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation [1, 2]. The Ca^{2+} effect is amplified by calmodulin and protein kinase C, which are related to a signal transduction in liver cells [3, 4]. Recently, it has been demonstrated that a novel Ca^{2+}-binding protein, which differs from calmodulin, is distributed in the hepatic cytosol of rats [5–9]. The name regucalcin was proposed for this Ca^{2+}-binding protein, which may regulate the Ca^{2+} effect on liver cell function; the protein can inhibit the activation of Ca^{2+}/calmodulin-dependent cyclic AMP phosphodiesterase [7], protein kinase C [8] and Ca^{2+} activated DNA fragmentation [9] due to binding of Ca^{2+}.

More recently, it has been reported that the expression of regucalcin mRNA, which is specific in the liver, is stimulated by a signaling through Ca^{2+}/calmodulin in rat liver [10, 11]. Hormonal regulation of the expression of regucalcin mRNA in rat liver, however, remains to be elucidated. Meanwhile, the supply of calcium induces an increase in serum calcium and a corresponding elevation of liver calcium in

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rats, which is regulated by a hypocalcemic hormone (calcitonin) from thyroid glands [12, 13]. Therefore, the present study was undertaken to clarify whether calcitonin (CT) can regulate the expression of hepatic regucalcin mRNA due to calcium administration in rats. It was found that hepatic regucalcin mRNA levels are increased by CT secretion following the oral administration of calcium in rats, and that the hormonal effect is mediated through Ca^{2+}.

Materials and methods

Hormone and chemicals. Synthetic [$Asu^{1,7}$] eel calcitonin (CT; 4000 MRC μ/mg) was supplied through the courtesy of Asahi Chemical Industry Co., Ltd. (Shizuoka, Japan). 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 (1.4 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 diethylprocarbonate (DEPC, Sigma) to inhibit RNase activity.

Animals. Wistar male rats (4-weeks old), 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.

Calcium administration. Calcium chloride was dissolved in sterile distilled water at concentrations of 25, 50 and 100 mg Ca per ml. These solutions were orally 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 0.25M sucrose solution and immediately removed and frozen at –80°C. Control animals received vehicle solution.

Surgical procedures. The thyroparathyroid gland complex was removed with fine forceps under light anesthesia with ether. Calcium chloride (100 mg Ca/100 g body weight) was orally administered with stomach tube 24 h after thyroparathyroidectomy (TPTX). CT (25, 50 and 100 MRC mU/100 g BW) was subcutaneously administered immediately after calcium administration. The rats were killed 60 min after the administration of calcium.

Calcium determination. The animals were bled by cardiac puncture under light anesthesia with ether. Blood samples obtained by cardiac puncture were centrifuged immediately after collection. The serum was separated and analysed immediately. Calcium in the serum was measured by atomic absorption spectrophotometry after precipitation with 10% tricholoracetic acid [14].

Liver was perfused with a cold 0.25 M sucrose solution after bleeding and removed immediately. The amount of calcium in liver tissues was determined by atomic absorption spectrophotometry after digestion with nitric acid [15]. The calcium content was expressed as μg per gram wet tissue.

Isolation of RNA. Total RNAs were prepared as described [10]. Liver and other tissues were quickly removed, rinsed with ice-cold 0.25M sucrose solution, and homogenized in buffer solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 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).

Northern blotting. Ten micrograms of total RNAs extracted from each tissue were electrophoresed in 1.2% agarose

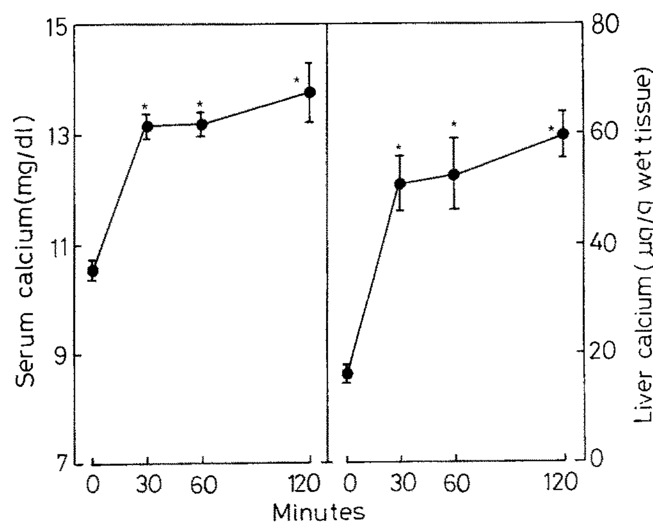

Fig. 1. Alteration of calcium concentration in the serum and liver of rats after a single oral administration of calcium. Calcium (100 mg/100 g body weight) was orally administered to rats, and the animals were sacrificed at 30, 60 and 120 min after the calcium administration. Each value is the mean ± SEM of five rats. * P< 0.01, as compared with the control (zero time) value.

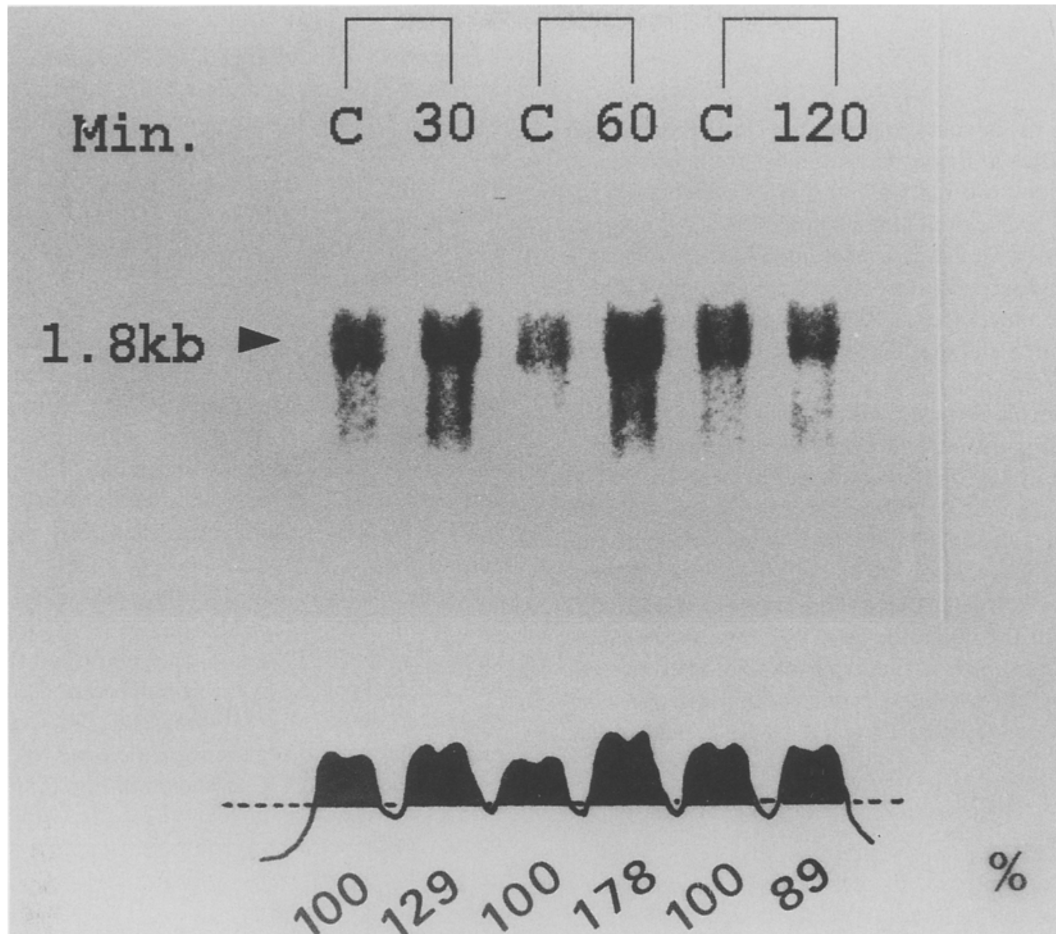

Fig. 2. Alteration of regucalcin mRNA levels in the liver of rats after a single oral administration of calcium. Calcium (100 mg/100 g body weight; BW) was orally administered to rats, and the animals were sacrificed at 30, 60 and 120 min after the calcium administration. Control animals (C) received an equivalent volume of the distilled water. Total RNA (10μg) 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 density of the autoradiographic data was quantified by densitometer scanning. The result shows one of four experiments with separate rats.

denaturing gels containing 2.2 M formaldehyde in MOPS buffer (pH 7.0, 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 1.4 kb, *KpnI-PstI* insert) was labelled 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 polyvinylprrolidine) and 0.5% SDS with ^{32}P-labelled 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 x 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).

Statistical methods The significance of the difference between the values was estimated by Student's *t*-test. P values less than 0.05 were considered to indicate a statistically significant difference.

Results

The alteration of calcium concentration in the serum and liver of rats after a single oral administration of calcium chloride solution (100 mg Ca/100 g body weight; BW) is shown in Fig. 1. The oral administration of calcium to rats produced a remarkable increase in serum calcium concentation and a corresponding elevation of liver calcium content. These increases were remarkable at 30 min after the oral administration of calcium, and those levels were maintained during 120 min.

The alteration of regucalcin mRNA in the liver of rats after a single oral administration of calcium (100 mg Ca/100 g BW) is shown in Fig. 2. Rats were sacrificed at 30, 60 and 120 min after the administration. At 60 min after calcium administration, liver regucalcin mRNA was markedly increased; the level was about 180% of the control level. Subsequently, the mRNA level began to decrease, and it reduced to about 90% 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 ug RNA per mg wet liver tissue.

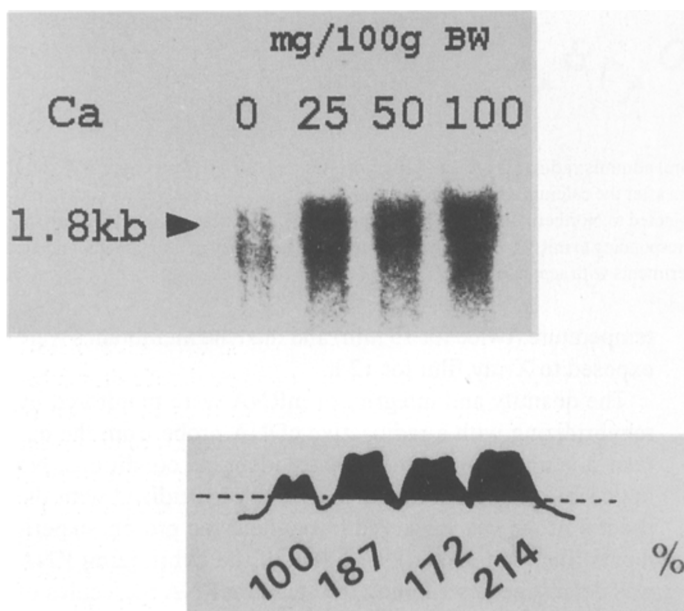

Fig. 3. Effect of increasing doses of calcium on regucalcin mRNA levels in the liver of rats. Animals received a single oral administration of calcium (25, 50 and 100 mg/100 g body weight; BW), and 60 min later they were sacrificed by bleeding. Control animals (0) received an equivalent volume of the 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.

The effect of increasing doses of calcium (25, 50 and 100 mg/100 g BW) on regucalcin mRNA in the liver of rats is shown in Fig. 3. The liver was removed 60 min after a single oral administration of calcium to rats. The effect of calcium administration on hepatic regucalcin mRNA was dose-dependent. Higher doses (100 mg Ca/100 g BW) produced greater mRNA levels. This dose produced a two-fold increase over control levels.

Calcitonin (CT), which has a hypocalcemic effect, is secreted from thyroid glands due to an increase in serum calcium concentration [12, 16]. Then, whether endogenous CT participates in the expression of regucalcin mRNA in the liver of rats received a single oral administration of calcium was examined. The solution of calcium (100 mg/100 g BW) was orally administered 24 h after thyroparathyroidectomy (TPTX), and the animals were sacrificed 60 min after the administration. TPTX rats showed a lower serum calcium level; the level was 7.15 ± 0.18 mg/dl. This observation recognized the depletion of hormones from thryoparathyroid glands in rats. TPTX completely abolished the increase in liver calcium content by the calcium administration (Fig. 4). Moreover, the calcium (100 mg/100 g BW) administration induced increase in hepatic regucalcin mRNA level was not entirely seen by TPTX, as shown in Fig. 5.

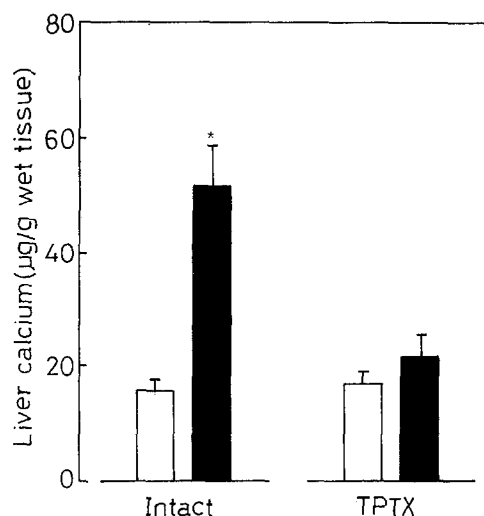

Fig. 4. Effect of thyroparathyroidectomy (TPTX) on calcium content in the liver of rats after a single oral administration of calcium. Calcium (100 mg/100 g body weight) was orally administered to intact (sham-operated) and TPTX rats, and 60 min later they were sacrificed by bleeding. Control animals received an equivalent volume of the distilled water. Each value is the mean ± SEM of five rats. * P < 0.01, as compared with the control value, □ ; Control ■ ; calcium administration.

Furthermore, the effect of exogenous CT on hepatic regucalcin mRNA levels was examined (Fig. 6).CT (25, 50 and 100 MRCmU/100 g BW) was subcutaneously administered immediately after the administation of calcium (100

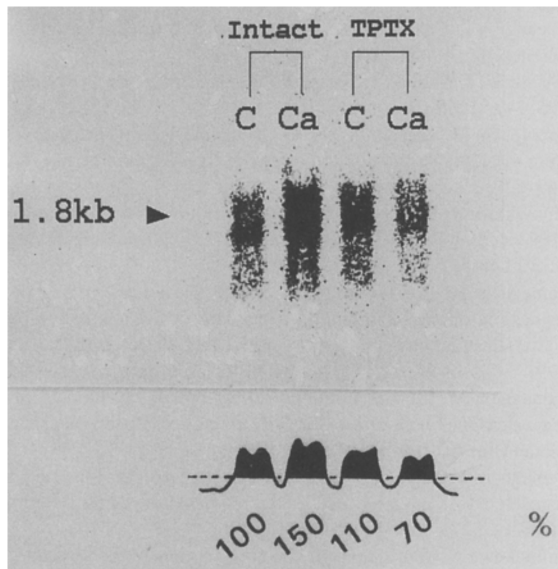

Fig. 5. Effect of thryoparathyroidectomy (TPTX) on regucalcin mRNA levels in the liver of rats after a single oral administration of calcium. Calcium (100 mg/100 g body weight) was orally administered to intact (sham-operated) and TPTX rats, and the animals were sacrificed at '60 min after the calcium administration. Control animals (C) 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.

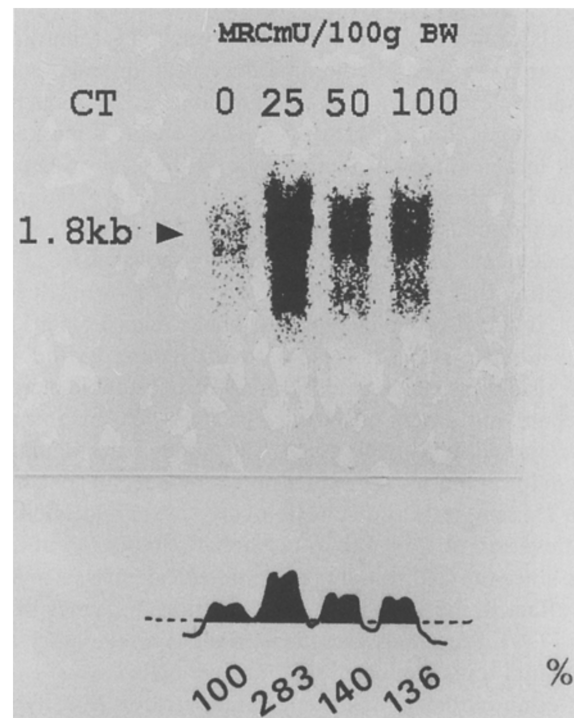

Fig. 6. Effect of increasing doses of calcitonin (CT) on regucalcin mRNA levels in the liver of rats. The thyroparathyroidectomized rats received a single oral administration of calcium (100 mg/100 g body weight; BW), and immediately the animals were subcutaneously administered CT (25, 50, and 100 MRC mU/100 g BW). The rats were sacrificed at 60 min after the administration of calcium. 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 results show one of four experiments with separate rats.

mg/100 g BW) to TPTX rats, and the animals were sacrificed 60 min after the calcium administration. The dose of 25 MRC mU/100 g BW CT produced a remarkable increase of regucalcin mRNA level in the liver; the level was about 280% of the control level. However, this increase was weakened with the higher doses of CT (50 and 100 MRC mU/100 g BW). Presumably, the physiological dose of CT was effective on the expression of hepatic regucalcin mRNA level. Now, the administration of CT (25 MRCmU/100 g BW) significantly increased liver calcium content (data not shown), as reported previously [13].

Discussion

A novel Ca^{2+}-binding protein regucalcin, isolated from rat liver cytosol, differs from other Ca^{2+}-binding protein such as calmodulin [3, 5–7]. Regucalcin has a reversible effect on liver cell function related to Ca^{2+} action. More recent investigations demonstrate that regucalcin mRNA is mainly distributed in the liver of rats, and that the expression is increased by a single intraperitoneal administration of calcium chloride [10]. Moreover, the calcium administration-increased expression of hepatic regucalcin mRNA was clearly inhibited by the administration of an antagonist of calmodulin, suggesting that the gene expression of regucalcin may be mediated through a Ca^{2+}/calmodulin-dependent protein kinase [11]. Regucalcin mRNA expression may not be involved in other protein kinases such as protein kinase C and cAMP-dependent protein kinase [11]. Thus, the role of signal transduction-related factors is suggested as the expression mechanism for mRNA of regucalcin in the liver of rats.

The physiological significance for the expression of hepatic regucalcin mRNA, however, is not clarified so far. The present study was undertaken to clarify hormonal regulation in the expression of regucalcin mRNA in the lvier of rats. A hypocalcemic hormone calcitonin (CT) is secreted from the thyroid glands due to an increase in serum calcium levels [16]. CT can stimulate Ca^{2+} inflow into liver cells [17], and the hormone induces the excretion of calcium into the he-

patic bile of rats [18]. In the present experiment, a single oral administration of calcium to rats produced a remarkable increase in the serum calcium concentration and a corresponding elevation of liver calcium content. Subsequently, hepatic regucalcin mRNA levels were elevated markedly. Thus, hypercalcemia induced a rapid increase in hepatic calcium content and regucalcin mRNA level. Hypercalcemia-induced increases in hepatic calcium content and regucalcin mRNA level were completely abolished by TPTX, suggesting that endogenous CT, which has a hypocalcemic effect, involves in the increase in hepatic regucalcin mRNA levels after the oral administration of calcium. By the way, the oral administration of calcium resulted in a rapid increase of hepatic regucalcin mRNA levels. However, this increase was returned to control levels. There may be a regulating system to suppress over-expression of regucalcin gene in rat liver. This suggests a cell physiological role of regucalcin for the elevation of Ca^{2+} due to hormonal stimulation in liver cells, since the Ca^{2+}-binding protein regucalcin has a reversible effect in the action of Ca^{2+} on various enzymes in the cells [7–9]. Presumably, regucalcin plays a regulatory role in a signal transduction of Ca^{2+} in liver cells.

In conclusion, it has been demonstrated that hypercalcemia-stimulated secretion of CT, which has a hypocalcemic effect, induces an increase in hepatic regucalcin mRNA levels mediated through Ca^{2+} in liver cells. This finding may support that regucalcin is involved in the regulation of liver calcium metabolism following the supply of calcium.

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