Volume 316, number 1, 79–84 FEBS 12017 January 1993

Expression of hepatic calcium-binding protein regucalcin mRNA is mediated through Ca^{2+}/calmodulin in rat liver

Noriaki Shimokawa and Masayoshi Yamaguchi

Laboratory of Metabolism and Endocrinology, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka-city 422, Japan

Received 7 October 1992; revised version received 2 December 1992

The effect of signal transduction-related factors was investigated to clarify the expression mechanism for mRNA of the hepatic Ca^{2+}-binding protein regucalcin in the liver of rats. The change of regucalcin mRNA levels was analyzed by Northern blotting using liver regucalcin cDNA (0.6 kb). A single intraperitoneal administration of calcium chloride (15 mg Ca^{2+}: 0.374 mmol/100 g body weight) to rats induced a remarkable increase of regucalcin mRNA in liver; the level was about 170% of controls at 30 min after administration. This increase was completely inhibited by simultaneous administration of trifluoperazine (5.0 mg/100 g), an antagonist of calmodulin. On the other hand, a single intraperitoneal administration of phorbol ester or dibutyryl cAMP (10–1,000 μg/100 g) did not cause a significant alteration of hepatic regucalcin mRNA levels. Also, administration of zinc, copper and cadmium (0.374 mmol of metal ion/100 g) did not have an appreciable effect on hepatic regucalcin mRNA levels. These findings demonstrate that the expression of hepatic regucalcin mRNA is mediated through Ca^{2+}/calmodulin.

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

1. INTRODUCTION

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions, and its part in liver metabolism has been demonstrated. Liver metabolism is regulated by an increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation. The Ca^{2+} effect is amplified by calmodulin and protein kinase C [1,2].

On the other hand, it has been recognized that a novel Ca^{2+}-binding protein, which differs from calmodulin, is distributed in the hepatic cytosol of rats [3–9]. The name regucalcin was proposed for this Ca^{2+}-binding protein, which may regulate the Ca^{2+} effect on liver cell function. Regucalcin has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells. 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+}.

More recently, we have reported that the expression of regucalcin mRNA is specific in the liver of various tissues and that it is increased by the administration of calcium chloride to rats [10]. Therefore, the present investigation was undertaken to clarify a possible regulatory mechanism for the expression of regucalcin mRNA by Northern blot analyses with hepatic regucalcin cDNA as a probe. It was found that the calcium administration-induced increase in rat hepatic mRNA levels is completely inhibited by treatment with an antagonist of calmodulin, trifluoperazine.

Correspondence address: M. Yamaguchi, Laboratory of Metabolism and Endocrinology, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka-city 422, Japan.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Deoxycytidine 5′-[α-^{32}P]triphosphate ([^{32}P]dCTP); 110 Tbq/mmol and nylon membranes (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 was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Trifluoperazine dimaleate (TFP), phorbol 12-myristate 13-acetate (PMA), and dibutyryl cyclic-adenosine 3′,5′-monophosphate sodium (dibutyryl cAMP) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride and other reagents were purchased from 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*

Male Wistar rats, weighing 80–100 g, purchased from Japan SLC Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid, Oriental Yeast Co., Ltd., Tokyo) containing 57.5% carbohydrate, 1.1% calcium and 1.1% phosphorus, and distilled water, ad libitum.

2.3. *Administration procedure*

$CaCl_2$, $ZnSO_4$, $CuSO_4$ and $CdCl_2$ were dissolved in sterile distilled water at a concentration of 0.374 mmol metal ions per ml. Also, TFP

(5.0 mg/ml), PMA (10, 100 and 1,000 μg/ml), and dibutyryl cAMP (10, 100 and 1,000 μg/ml) were dissolved in sterile distilled water. These solutions (1.0 ml/100 g body weight) were intraperitoneally administered to rats. At 30 min after the 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*

Hepatic total RNAs were prepared as described [11]. Liver was 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) with 3 V/cm for 3 h [12]. The electrophoresed gels were transferred to nylon membranes by blotting [12]. Part of regucalcin cDNA (the 0.6 kb, *Kpn*I–*Pst*I insert) was labeled with [^{32}P]dCTP by random primers with the DNA polymerase Klenow fragment [13]. 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, 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, each for 15 min), followed by 0.1 × SSPE and 0.1% SDS at room temperature (twice, each for 15 min), and then the membranes were exposed to X-ray film for 12 h.

Quantity and integrity of mRNA were monitored by rehybridizing with a radioactive cDNA probe from 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 present experiments (data not shown). The size of the hybridizing RNA was determined by running the standard RNA molecules of known sizes in parallel. The density of the autoradiographic data was quantified by densitometer scanning (Dual-wavelength Flying-spot Scanner, CS-9000, Shimadzu Co., Japan).

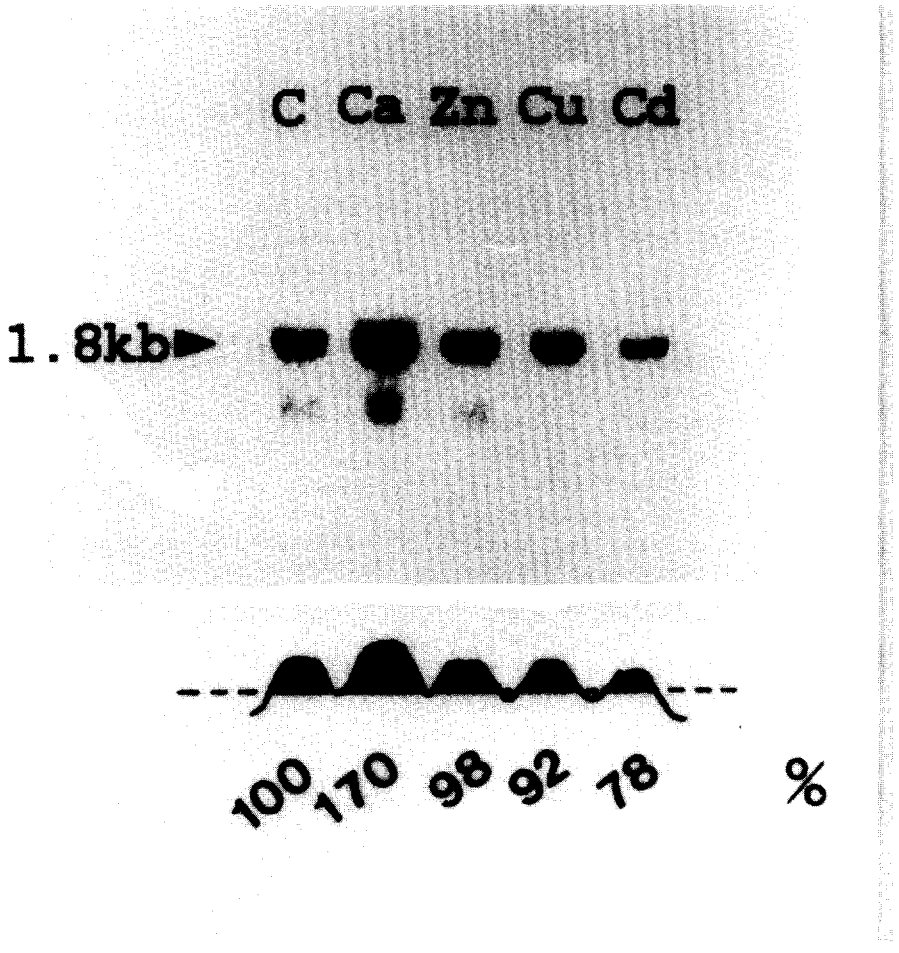

Fig. 1. Effect of the administration of various metals on regucalcin mRNA levels in the liver of rats. Animals received a single intraperitoneal administration of the indicated metal ion (0.374 mmol/100 g body weight), and 30 min later they were sacrificed by bleeding. Control animals (C) received an equivalent volume of distilled water. Total RNA (10 μg) isolated from the liver were subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. The arrowhead indicates hybridizing bands corresponding to mRNA encoding regucalcin. The figure shows one of four experiments with separate rats.

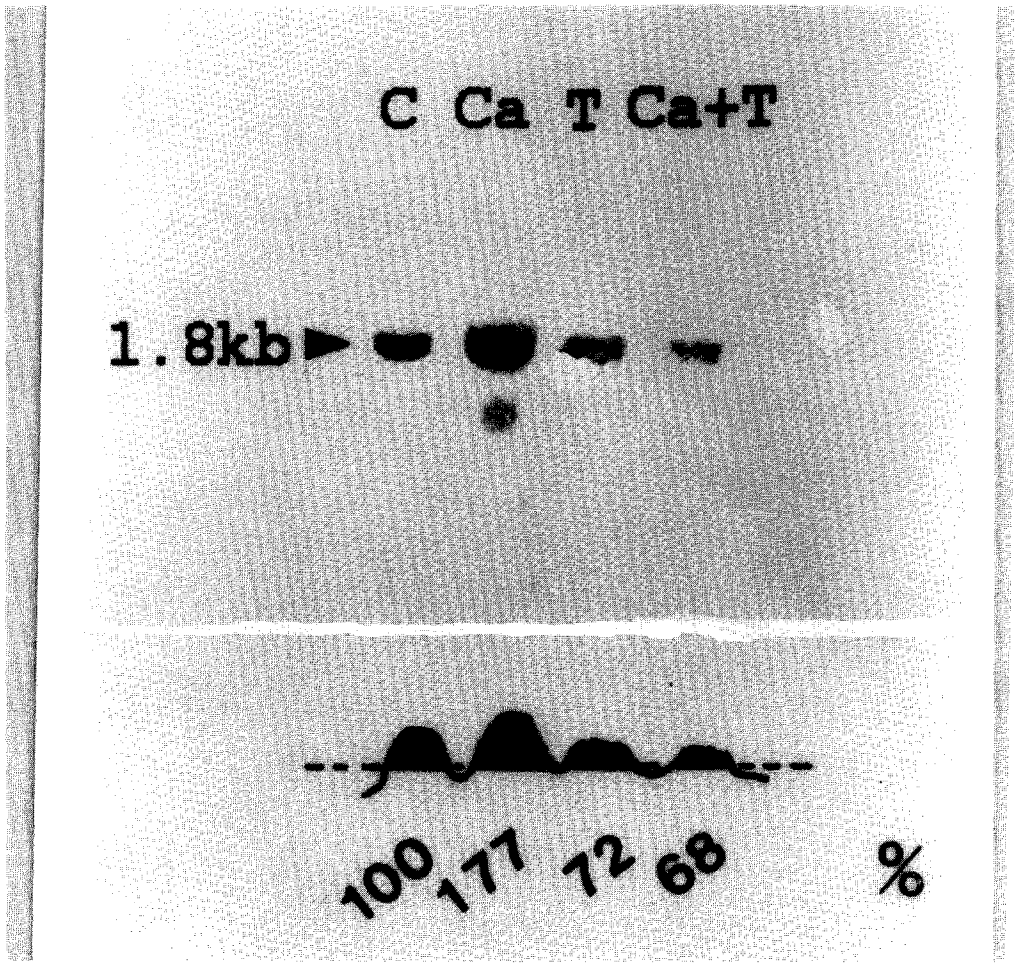

Fig. 2. Effect of the calmodulin antagonist (TFP) administration on regucalcin mRNA levels in the liver of rats. Animals received a single intraperitoneal administration of calcium (Ca, 15 mg/100 g body weight) or TFP (T, 5.0 mg/100 g) or both (Ca+T), and 30 min later they were sacrificed by bleeding. Control animals (C) received an equivalent volume of distilled water. Total RNA (10 μg) isolated from the liver were subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. The arrowhead indicates hybridizing bands corresponding to mRNA encoding the regucalcin. The figure shows one of four experiments with separate rats.

3. RESULTS

The effect of various metals on regucalcin mRNA in the liver of rats is shown in Fig. 1. The various metals (calcium, zinc, copper, and cadmium; 0.374 mmol/100 g body weight) were intraperitoneally administered to rats, and the animals were sacrificed 30 min after the administration. Previous experiments showed that the hepatic regucalcin mRNA level is markedly increased by the administration of calcium (0.374 mmol/100 g body weight) [9]. Hepatic regucalcin mRNA was markedly increased by calcium administration; the level was about 170% of control. Meanwhile, the expression of regucalcin mRNA was not appreciably altered by the administration of zinc, copper and cadmium. Thus, the expression of liver regucalcin mRNA was unique for calcium out of the various metals used.

The effect of an antagonist of calmodulin (TFP) on regucalcin mRNA in the liver of rats is shown in Fig. 2. The administration of calcium (15 mg (0.374 mmol)/100 g body weight) caused a remarkable increase in the regucalcin mRNA level. When calcium (15 mg/100 g body weight) and TFP (5.0 mg/100 g body weight) were administered simultaneously, TFP completely blocked the effect of calcium. The administration of TFP alone (5.0 mg/100 g body weight) caused a slight decrease of the hepatic regucalcin mRNA level. Thus, the calcium administration-increased expression of hepatic regucalcin mRNA was mediated through calmodulin.

Then, the effect of other signal transduction related factors was examined. The effect of PMA, a phorbol ester which activates protein kinase C (PKC), on the expression of regucalcin mRNA in the liver of rats is shown in Fig. 3. The solution of PMA (10, 100 and 1,000 μg/100 g body weight) was administered intraperitoneally to rats, and the animals were sacrificed 30 min

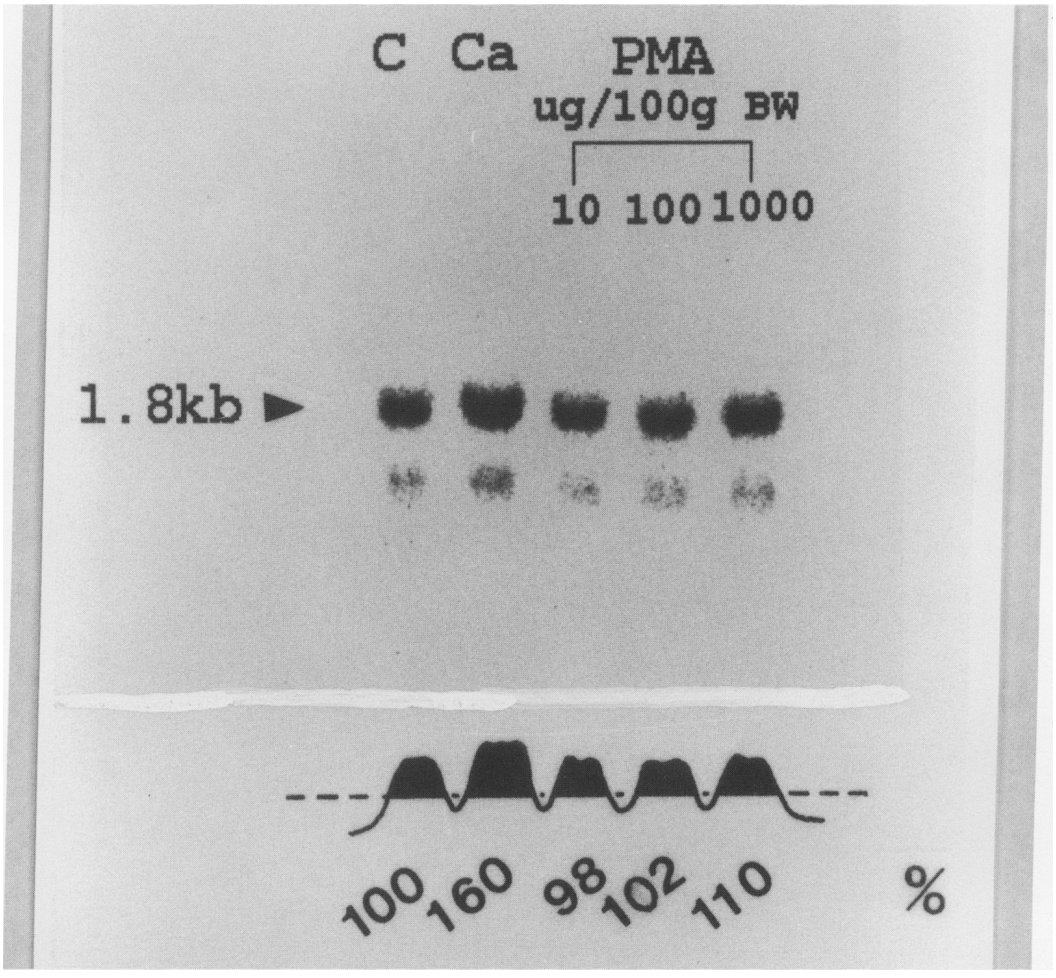

Fig. 3. Effect of increasing doses of PMA on regucalcin mRNA levels in the liver of rats. Animals received a single intraperitoneal administration of calcium (Ca, 15 mg/100 g body weight) or PMA (10, 100 and 1,000 μg/100 g), and 30 min later they were sacrificed by bleeding. Control animals (C) received an equivalent volume of distilled water. Total RNAs (10 μg) isolated from the liver were subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. The arrowhead indicates hybridizing bands corresponding to mRNA encoding the regucalcin. The figure shows one of four experiments with separate rats.

after the administration. The expressional change in hepatic regucalcin mRNA by the administration of PMA was not observed at any doses.

Moreover, the effect of dibutyryl cAMP on the expression of regucalcin mRNA in rat liver was examined, and the results are shown in Fig. 4. The expression of hepatic regucalcin mRNA was not altered by the administration of dibutyryl cAMP (10, 100 and 1,000 μg/100 g body weight), as compared with that of control rats.

4. DISCUSSION

A novel calcium-binding protein regucalcin, isolated from rat liver cytosol, differs from other calcium-binding protein such as calmodulin [3–9]. Regucalcin can regulate the Ca^{2+} effect on liver cell function. The expression of the regucalcin gene, however, is not fully clarified so far. More recent investigations demonstrate that regucalcin mRNA is mainly distributed in the liver of rats, and that the expression is increased by the administration of calcium; the mRNA level was clearly increased at the early time point (within 30 min) of calcium administration [10]. Presumably, regucalcin mRNA expression is stimulated by increasing calcium in liver cells. Furthermore, the present study was undertaken to clarify a possible regulatory mechanism of the expression of regucalcin mRNA in the liver of rats. Regucalcin mRNA levels in rat liver was estimated by Northern blot analyses with a hepatic regucalcin cDNA probe.

First, the effect of various metal ions on the expression of regucalcin mRNA in rat liver was examined. A single intraperitoneal administration of zinc, copper or cadmium with a dose of 0.374 mmol/100 g body weight did not cause an appreciable increase of regucalcin

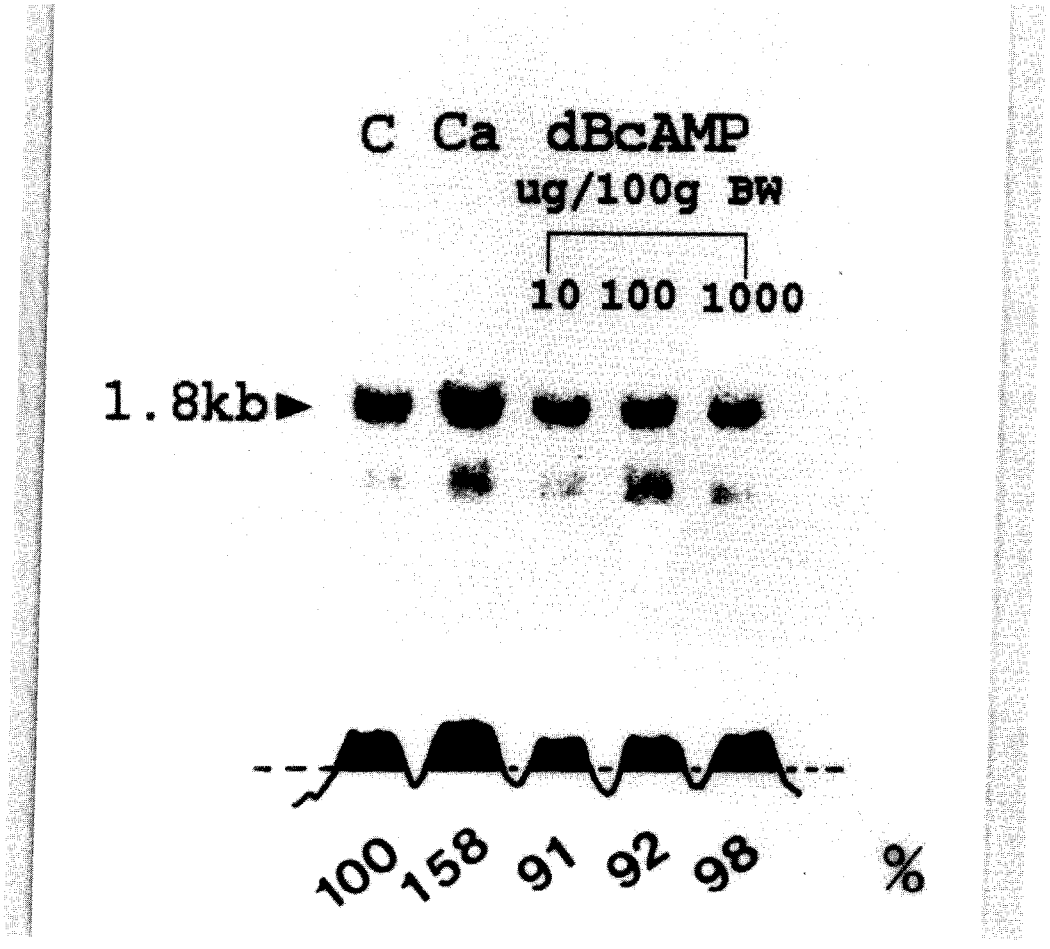

Fig. 4. Effect of increasing doses of dibutyryl cAMP on regucalcin mRNA levels in the liver of rats. Animals received a single intraperitoneal administration of calcium (Ca, 15 mg/100 g body weight) or dibutyryl cAMP (10, 100 and 1,000 μg/100 g), and 30 min later they were sacrificed by bleeding. Control animals (C) received an equivalent volume of distilled water. Total RNAs (10 μg) isolated from the liver were subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. The arrowhead indicates hybridizing bands corresponding to mRNA encoding the regucalcin. The figure shows one of four experiments with separate rats.

mRNA levels in the liver of rats, while calcium administration induced a remarkable increase in hepatic regucalcin mRNA levels. Thus, regucalcin mRNA expression was uniquely stimulated by calcium out of the various metals used. Then, we further tested the effect of TFP, an antagonist of calmodulin, on the expression of regucalcin mRNA in rat liver. It is known that TFP inhibits various enzymes and biological processes that are stimulated by calmodulin in the presence of Ca^{2+} [14]. Also, calmodulin is known to exist in rat liver nuclei [15]. Present data show that TFP completely blocked the expression of hepatic regucalcin mRNA as increased by the administration of calcium. This suggests that regucalcin mRNA expression is activated by Ca^{2+}/calmodulin. Presumably, the expression of regucalcin mRNA in rat liver is mediated through a certain intermediate protein which is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase [16]. The mechanism related to phosphorylation of intermediate protein in liver nuclei remains to be elucidated.

Phorbol myristate acetate (PMA) can activate a protein kinase C (PKC) in the same way as diacylglycerol [17]. PKC is located in rat liver nuclei [18]. PMA was intraperitoneally administered in rats to see whether PKC activation can regulate hepatic regucalcin mRNA levels. The administration of PMA (10–1,000 μg/100 g body weight) to rats did not cause an appreciable alteration of the regucalcin mRNA level in the liver. Presumably, the expression of hepatic regucalcin mRNA may not be mediated through protein kinase C.

The palindromic consensus sequence (TGACGTCA), which functions as a cAMP-responsive element (CRE), was found in the 5′-flanking region in the human glycoprotein hormone α-subunit gene [19]. CRE-binding protein (CREB), which binds to a specific sequence (TGACGTCA), is phosphorylated by cAMP-depend-

ent protein kinase and the CRE-phosphorylated CREB complex stimulates transcription [20]. Then the effect of dibutyryl cAMP on the expression of regucalcin mRNA in rat liver was also examined. Dibutyryl cAMP was intraperitoneally administered to rats at a dose of 10–1,000 μg/100 g body weight. The administration of dibutyryl cAMP did not result in alteration of the expression level of mRNA encoding regucalcin in the liver, suggesting that the gene encoding regucalcin does not have CRE.

In conclusion, the present study using Northern blot analyses demonstrates that the calcium administration-increased expression of hepatic regucalcin mRNA was clearly inhibited by the administration of an antagonist of calmodulin (TFP), suggesting that the gene expression of regucalcin may be mediated through a Ca^{2+}/calmodulin-dependent protein kinase. Regucalcin mRNA expression may not be involved in other protein kinases such as protein kinase C and cAMP-dependent protein kinase.

REFERENCES

[1] Williamson, J.R., Cooper, R.H. and Hoek, J.B. (1981) Biochim. Biophys. Acta 639, 243–295.
[2] Nishizuka, Y. (1984) Nature 308, 693–698.
[3] Yamaguchi, M. and Yamamoto, T. (1978) Chem. Pharm. Bull. 26, 1915–1918.
[4] Yamaguchi, M. and Sugii, K. (1981) Chem. Pharm. Bull. 29, 567–570.
[5] Yamaguchi, M. and Yoshida, H. (1985) Chem. Pharm. Bull. 33, 4489–4493.
[6] Yamaguchi, M. and Mori, S. (1990) Biochem. Med. Metab. Biol. 43, 140–146.
[7] Mori, S. and Yamaguchi, M. (1990) Chem. Pharm. Bull. 38, 2216–2218.
[8] Yamaguchi, M. and Tai, H. (1991) Mol. Cell. Biochem. 106, 25–30.
[9] Yamaguchi, M. and Sakurai, T. (1991) FEBS Lett. 279, 281–284.
[10] Shimokawa, N. and Yamaguchi, M. (1992) FEBS Lett. 305, 151–154.
[11] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T., in: Molecular Cloning (2nd Edn.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, pp. 7.39–7.52.
[13] Feinberg, A. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[14] Weiss, B. and Wallace, T.L., in: Calcium and Cell Function (W.Y. Cheung, Ed.), Academic Press, New York, 1980, pp. 329–379.
[15] Bachs, O. and Carafolli, E. (1987) J. Biol. Chem. 262, 10786–10790.
[16] Connelly, P.A., Sisk, R.B., Schulman, H. and Garrison, J.C. (1987) J. Biol. Chem. 262, 10154–10163.
[17] Wasserman, R.H., in: Calcium in Human Biology (B.E.C. Nordin, Ed.), Springer, Berlin, 1988, pp. 385–419.
[18] Rogue, P., Labourdette, G., Masmoudi, A., Yoshida, Y., Huang, F.L., Huang, K.-P., Zwiller, J., Vincendon, G. and Malviya, A.N. (1990) J. Biol. Chem. 265, 4161–4165.
[19] Silver, B.J., Bokar, J.A., Virgin, J.B., Vallen, E.A., Milsted, A. and Nilson, J.H. (1987) Proc. Natl. Acad. Sci. USA 84, 2198–2202.
[20] Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L. and Habener, J.F. (1988) Science 242, 1430–1433.