Effect of the Calcium-Binding Protein Regucalcin on the Ca^{2+} Transport System in Rat Liver Microsomes: The Protein Stimulates Ca^{2+} Release

Masayoshi YAMAGUCHI* and Seiichi MORI

Department of Environmental Biochemistry and Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 395 Yada, Shizuoka-city 422, Japan. *Received March 14, 1989*

The effect of the calcium-binding protein regucalcin on the Ca^{2+} transport system in the liver microsomes from fed rats was investigated. Ca^{2+} transport was assayed by the method of Millipore filtration to estimate microsomal $^{45}Ca^{2+}$ accumulation following addition of 10 mM adenosine triphosphate (ATP). $^{45}Ca^{2+}$ uptake was retarded by the presence of regucalcin (1.0—4.0 μM). This retardation was remarkable at 1 min after regucalcin addition, while appreciable retardation was no longer seen at 5 min. Regucalcin (2.0 μM)-induced retardation of $^{45}Ca^{2+}$ uptake was prevented by the presence of calmodulin (5 μg/ml). Calmodulin alone (1 and 5 μg/ml) caused a significant increase in $^{45}Ca^{2+}$ uptake at 3 min after the start of incubation. Also, regucalcin (2.0 μM)-induced retardation of $^{45}Ca^{2+}$ uptake was completely blocked by the presence of a Ca^{2+}-trapping agent, oxalate (3 mM). On the other hand, $^{45}Ca^{2+}$, which accumulated in microsomes during 5 min after ATP addition, was markedly released by the addition of regucalcin. This release was dose-dependent (0.5—4.0 μM). Guanosine triphosphate (GTP; 10—100 μM) caused a significant release of $^{45}Ca^{2+}$ from the microsomes. The presence of regucalcin (2.0 μM) further enhanced the GTP effect. Regucalcin (2.0 μM)-induced release of $^{45}Ca^{2+}$ was not blocked by the presence of the protein thiol-protecting agent dithiothreitol (0.1 mM). The presence of oxalate (3 mM) completely blocked the effect of regucalcin on $^{45}Ca^{2+}$ release from the microsomes. These results indicate that regucalcin stimulates Ca^{2+} release from liver microsomes, and that the protein retards the microsomal Ca^{2+} uptake. The present study suggests that regucalcin can regulate the Ca^{2+} transport system in rat liver microsomes.

Keywords regucalcin; calcium-binding protein; calcium; calcium uptake; calcium release; calmodulin; GTP; rat liver microsome

It has been established that liver metabolism is regulated by increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation.[1,2] The Ca^{2+} effect is modulated through calmodulin, a calcium-binding protein, in liver cells.[3,4] Ca^{2+} plays an important role in the regulation of liver cell function. In recent years, we have reported that a calcium-binding protein (regucalcin), which differs from calmodulin, is distributed in the hepatic cytosol of rats.[5-7] The molecular weight of regucalcin isolated from rat liver cytosol was estimated to be 28800, and the Ca^{2+} binding constant was found to be $4.19 \times 10^5\ M^{-1}$ by equilibrium dialysis.[6] This novel protein has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells.[7-10] Regucalcin may play a cell physiological role in the regulation of liver cell function related to Ca^{2+}.

More recently, it has been found that regucalcin can directly activate (Ca^{2+}–Mg^{2+})-adenosine triphosphatase, which functions as a Ca^{2+} pump in rat liver plasma membranes.[11] It is suggested that regucalcin may play a role in the stimulation of the extrusion of increased cytosolic Ca^{2+} in liver cells.[11] Presumably, regucalcin participates in the regulation of Ca^{2+} homeostasis in liver cells.

On the other hand, it has been demonstrated that inositol 1,4,5-triphosphate, which is a second messenger for hormonal stimulation, can release Ca^{2+} from rat liver microsomes.[12,13] The present investigation was therefore undertaken to clarify the effect of regucalcin on the Ca^{2+} transport system in rat liver microsomes. It was found that regucalcin stimulates Ca^{2+} release from the microsomes. The present finding supports the view that regucalcin plays a role as a regulatory protein for Ca^{2+} in liver cells.

Materials and Methods

Animals Male Wistar rats, weighing 100—120 g, were used. They were obtained commercially from Japan SLC. Inc., 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 in the cytosol fraction of rat liver 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.[5]

Preparation of Hepatic Microsomes Rats were killed by cardiac puncture, and the liver was perfused with ice-cold 250 mM sucrose solution, immediately cut into small pieces, suspended 1 : 9 in the homogenization medium containing 250 mM sucrose, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 1.0 mM ethyleneglycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 1 mM dithiothreitol (DTT), pH 7.2 and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle.[14] The homogenate was centrifuged at $1000 \times g$ for 10 min to remove nuclei, unbroken cells and cell debris. The resultant supernate was centrifuged at $7700 \times g$ for 20 min to remove the mitochondrial fraction. The postmitochondrial supernate was then centrifuged at $110000 \times g$ for 60 min to sediment the microsomal fraction. The microsomal fraction was resuspended in 120 mM KCl, 10 mM Hepes, pH 6.8, to a final protein concentration of 20—30 mg/ml.

$^{45}Ca^{2+}$ Uptake $^{45}Ca^{2+}$ uptake was measured by the Millipore filtration technique.[15] About 170—190 μg of protein/ml was incubated for 1 min at 37 °C in 1 ml of medium containing 100 mM KCl, 20 mM Hepes, 1 mM NaN_3, 1 mM $MgCl_2$, 1 μM ruthenium red and 100 μM $CaCl_2$ containing $^{45}Ca^{2+}$ (5.0 μCi), pH 6.8. At a designated time after addition of 10 mM adenosine triphosphate (ATP; adjusted to pH 6.8 with KOH) to initiate energy-dependent Ca^{2+} uptake, a 100 μl sample was filtered through a 0.22 μm pre-wetted Millipore filter. The precipitate was washed with 120 mM KCl/100 mM Hepes, pH 6.8, transferred to a scintillation vial and counted for radioactivity. $^{45}Ca^{2+}$ uptake is expressed as nmol of $^{45}Ca^{2+}$ accumulated per mg protein of the microsomes.

$^{45}Ca^{2+}$ Release Isolated rat liver microsomes were incubated in the presence of 100 μM Ca^{2+} and 10 mM ATP for 5 min at 37 °C with 5.0 μCi of $^{45}Ca^{2+}$ in the Ca^{2+} uptake assay buffer,[16] and then regucalcin (0.5—4.0 μM) was added to the incubation mixture. At a designated time after the addition of regucalcin, a 100 μl sample was filtered through a 0.22 μm pre-wetted Millipore filter to determine the amount of $^{45}Ca^{2+}$ remaining in the vesicles. The precipitate on the filter after filtration was washed with 120 mM KCl/10 mM Hepes, pH 6.8, and transferred to scintillation vial. $^{45}Ca^{2+}$ remaining in the microsomes is expressed as nmol of $^{45}Ca^{2+}$ per mg protein of the microsomes.

Analytical Methods Protein concentration was determined by the method of Lowry *et al.*[17] Calcium content in the microsomes was

determined by atomic absorption spectrophotometry after digestion with nitric acid, and expressed as amount of calcium (nmol) per mg protein of the microsomes.

Reagents $^{45}CaCl_2$ (specific activity 44.56 mCi/mg) used was obtained from New England Nuclear (Boston, Mass., U.S.A.). Other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and were dissolved in distilled water then passed through an ion-exchange resin to remove metal ions.

Statistical Methods The significance of differences between values was estimated by using Student's t test; p values of less than 0.05 were considered to indicate statistically significant differences.

Results

Effect of Regucalcin on $^{45}Ca^{2+}$ Uptake by Liver Microsomes The microsomes isolated from rat liver contained total calcium of 4.20 ± 0.10 nmol per mg of microsomal protein, as determined by atomic absorption spectrophotometry. The microsomes were incubated for 1 min in medium containing $^{45}Ca^{2+}$ and regucalcin (2.0 μM), and then ATP (10 mM) was added. $^{45}Ca^{2+}$ uptake by the microsomes increased rapidly during 1 min after ATP addition, and it was saturated at 2 min (Fig. 1). The presence of regucalcin (2.0 μM) caused a significant retardation of $^{45}Ca^{2+}$ uptake by the microsomes (Fig. 1). This retardation was seen at 0.5 min after ATP addition and reached a maximum at 2 min.

The effect of increasing concentrations of regucalcin (0.5, 1.0, 2.0 and 4.0 μM) on $^{45}Ca^{2+}$ uptake by the microsomes is shown in Fig. 2. The microsomes were incubated for 1 min after the addition of 10 mM ATP. The microsomal $^{45}Ca^{2+}$ uptake was significantly retarded by the presence of 1.0 μM regucalcin. At greater concentrations, the regucalcin effect was remarkable.

The effect of calmodulin on $^{45}Ca^{2+}$ uptake by the microsomes is shown in Fig. 3. The presence of calmodulin (1.0 and 5.0 μg/ml) caused a significant increase in the microsomal $^{45}Ca^{2+}$ uptake at 3 min after the addition of 10 mM ATP, though the calmodulin effect was not seen at 1 min. The effect of regucalcin (2.0 μM) to retard $^{45}Ca^{2+}$ uptake by the microsomes was blocked by the presence of calmodulin.

The effect of regucalcin on $^{45}Ca^{2+}$ uptake by the microsomes in the presence of the Ca^{2+} trapping agent oxalate is shown in Fig. 4. The microsomes were incubated for 1 and 3 min after the addition of 10 mM ATP with or without 3 mM oxalate. The microsomal $^{45}Ca^{2+}$ uptake was slightly increased by the presence of 3 mM oxalate. The effect of regucalcin (2.0 μM) to retard the microsomal $^{45}Ca^{2+}$ uptake was not fully apparent in the presence of 3 mM oxalate.

Effect of Regucalcin on $^{45}Ca^{2+}$ Release from Liver Microsomes Isolated liver microsomes were incubated for 1 min in medium containing $^{45}Ca^{2+}$, and then 10 mM ATP was added and further incubated for 5 min. The micro-

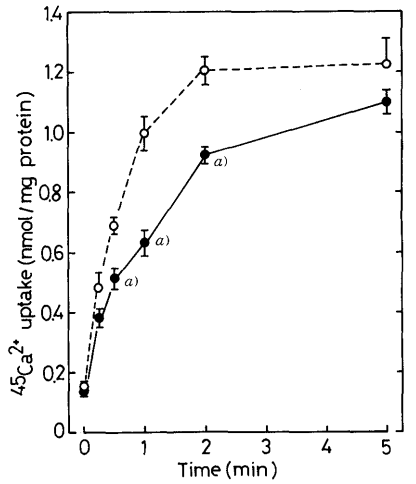

Fig. 1. Effect of Regucalcin on $^{45}Ca^{2+}$ Uptake by the Microsomes of Rat Liver

$^{45}Ca^{2+}$ uptake was measured as described in the experimental section. The amount at time zero represents $^{45}Ca^{2+}$ bound to the microsomes when incubated for 1 min before the addition of ATP. At zero time ATP was added to give a final concentration of 10 mM. Regucalcin was included in the incubation medium at a concentration of 2.0 μM. Each value represents the mean $\pm$ S.E.M. of 5 experiments. *a)* $p < 0.01$, as compared with the value without regucalcin. ○, none; ●, regucalcin.

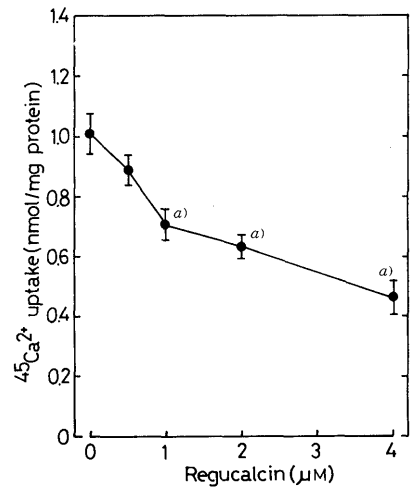

Fig. 2. Effect of Increasing Concentrations of Regucalcin on $^{45}Ca^{2+}$ Uptake by the Microsomes of Rat Liver

$^{45}Ca^{2+}$ uptake was measured as described in the experimental section. The microsomes were incubated for 1 min after the addition of ATP. Regucalcin was included in the incubation medium at a concentration of 0.5, 1.0, 2.0 or 4.0 μM. Each value represents the mean $\pm$ S.E.M. of 5 experiments. *a)* $p < 0.01$, as compared with the value without regucalcin.

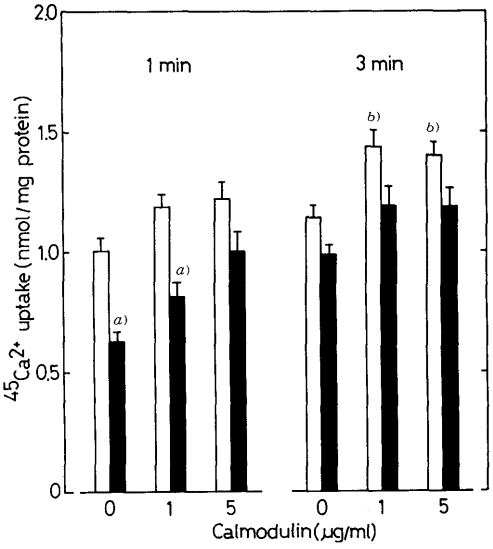

Fig. 3. Effect of Calmodulin on $^{45}Ca^{2+}$ Uptake by the Microsomes of Rat Liver

$^{45}Ca^{2+}$ uptake was measured as described in the experimental section. The microsomes were incubated for 1 and 3 min after the addition of ATP with or without 2.0 μM regucalcin. Calmodulin was included in the incubation medium at a concentration of 1 or 5 μg/ml. Each value represents the mean $\pm$ S.E.M. of 5 experiments. *a)* $p < 0.01$, as compared with the value without regucalcin. *b)* $p < 0.01$, as compared with the value of the control at zero time. □, none; ■, regucalcin.

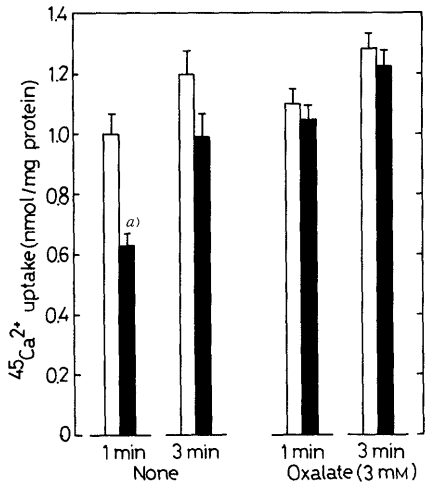

Fig. 4. Effect of Oxalate on Regucalcin-Induced Retardation of $^{45}Ca^{2+}$ Uptake by the Microsomes of Rat Liver

$^{45}Ca^{2+}$ uptake was measured as described in the experimental section. The microsomes were incubated for 1 and 3 min after the addition of ATP with or without 3.0 mM oxalate. Regucalcin was included in the incubation medium at a concentration of 2.0 μM. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without regucalcin. □, none; ■, regucalcin.

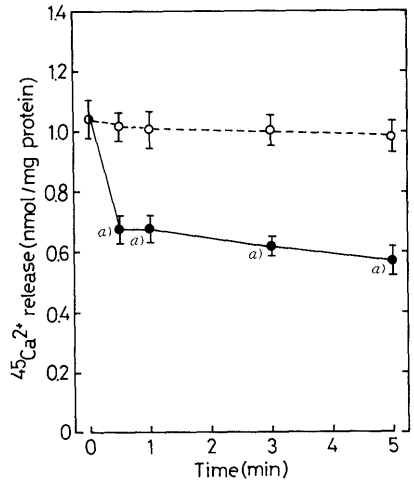

Fig. 5. Effect of Regucalcin on $^{45}Ca^{2+}$ Release from the Microsomes of Rat Liver

$^{45}Ca^{2+}$ release was measured as described in the experimental section. The amount at time zero represents $^{45}Ca^{2+}$ taken up by the microsomes when incubated for 5 min after the addition of ATP. At zero time regucalcin was added to give a final concentration of 2.0 μM. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without regucalcin. ○, none; ●, regucalcin.

somal $^{45}Ca^{2+}$ uptake was saturated by incubation for 5 min with 10 mM ATP. At this time, regucalcin was added to the incubation mixture. The addition of regucalcin (2.0 μM) caused a rapid release of $^{45}Ca^{2+}$ from the microsomes during 0.5 min (Fig. 5). The amount of $^{45}Ca^{2+}$ released by regucalcin was about 40% of $^{45}Ca^{2+}$ accumulated in the microsomes. $^{45}Ca^{2+}$ release from the microsomes caused by regucalcin was saturated at 3 min after the addition of regucalcin.

The effect of increasing concentrations of regucalcin (0.5—4.0 μM) on $^{45}Ca^{2+}$ release from liver microsomes is shown in Fig. 6. The stimulatory effect of regucalcin on $^{45}Ca^{2+}$ release from the microsomes was seen at 0.5 μM regucalcin, which did not have an appreciable effect on $^{45}Ca^{2+}$ uptake by the microsomes. The effect of regucalcin

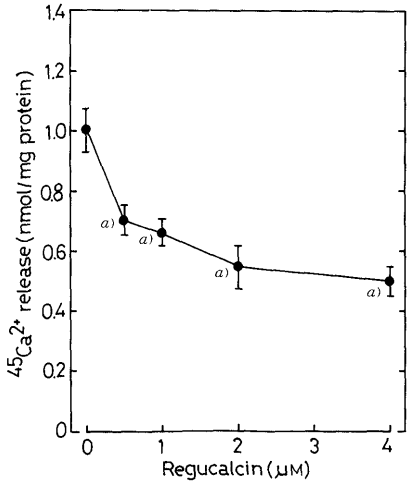

Fig. 6. Effect of Increasing Concentrations of Regucalcin on $^{45}Ca^{2+}$ Release from the Microsomes of Rat Liver

$^{45}Ca^{2+}$ release was measured as described in the experimental section. The microsomes were incubated for 5 min after the addition of ATP, and then regucalcin was added to give a final concentration of 0.5, 1.0, 2.0 or 4.0 μM. The microsomes were incubated for 3 min after the addition of regucalcin. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without regucalcin.

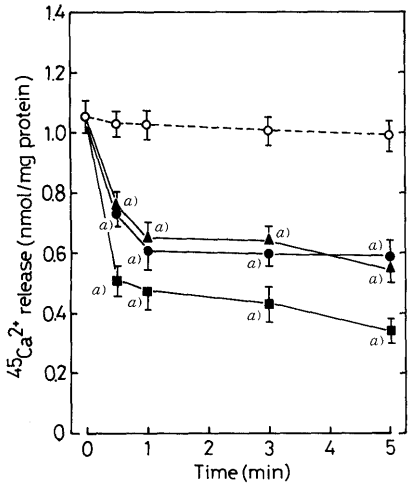

Fig. 7. Effect of GTP on $^{45}Ca^{2+}$ Release from the Microsomes of Rat Liver

$^{45}Ca^{2+}$ release was measured as described in the experimental section. The amount at time zero represents $^{45}Ca^{2+}$ taken up by the microsomes when incubated for 5 min after the addition of ATP. At zero time GTP was added to give a final concentration of 10, 25 or 100 μM. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without GTP. ○, none; ▲, 10 μM GTP; ●, 25 μM GTP; ■, 100 μM GTP.

on the microsomal $^{45}Ca^{2+}$ release was saturated at 2.0 μM regucalcin.

It has been reported that guanosine triphosphate (GTP) can stimulate Ca^{2+} release from isolated rat liver microsomes.[18,19] The effect of GTP on $^{45}Ca^{2+}$ release from the microsomes was examined in this study, and the result is shown in Fig. 7. Addition of GTP (10, 25 and 100 μM) caused a significant release of $^{45}Ca^{2+}$ from the microsomes at 0.5 min after GTP addition. On the other hand, the addition of both GTP and regucalcin caused an additive effect on $^{45}Ca^{2+}$ release from the microsomes (Fig. 8). In the presence of GTP (10, 25 and 100 μM), regucalcin (2.0 μM) further stimulated $^{45}Ca^{2+}$ release from the microsomes.

The effect of dithiothreitol, a protecting agent of thiol

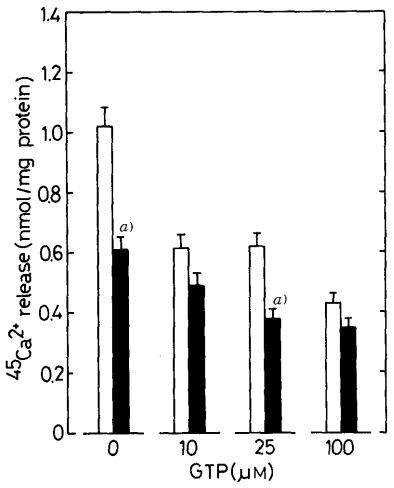

Fig. 8. Effect of Regucalcin on GTP-Induced Release of $^{45}Ca^{2+}$ from the Microsomes of Rat Liver

$^{45}Ca^{2+}$ release was measured as described in the experimental section. The microsomes were incubated for 5 min after the addition of ATP, and then GTP was added to give a final concentration of 10, 25 or 100 μM with or without 2.0 μM regucalcin. The microsomes were incubated for 3 min after the addition of regucalcin. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without regucalcin. □, none; ■, regucalcin.

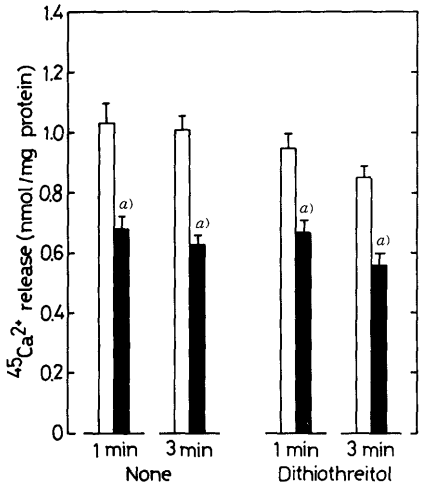

Fig. 9. Effect of Dithiothreitol on Regucalcin-Induced Release of $^{45}Ca^{2+}$ from the Microsomes of Rat Liver

$^{45}Ca^{2+}$ release was measured as described in the experimental section. The microsomes were incubated in the presence of 100 μM dithiothreitol for 5 min after the addition of ATP, and then regucalcin was added to give a final concentration of 2.0 μM. The microsomes were incubated for 1 or 3 min after the addition of regucalcin. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without regucalcin. □, none; ■, regucalcin.

groups of membrane proteins, on regucalcin-induced release of $^{45}Ca^{2+}$ from the microsomes is shown in Fig. 9. The presence of 100 μM dithiothreitol did not cause an appreciable modification of the microsomal $^{45}Ca^{2+}$ release induced by 2.0 μM regucalcin.

When the microsomes were incubated in medium containing 10 mM ATP and 3 mM oxalate, the microsomal $^{45}Ca^{2+}$ uptake was enhanced markedly (Fig. 10). In the presence of the Ca^{2+} trapping agent oxalate, the effect of regucalcin (2.0 μM) to stimulate $^{45}Ca^{2+}$ release from the microsomes was not seen (Fig. 10).

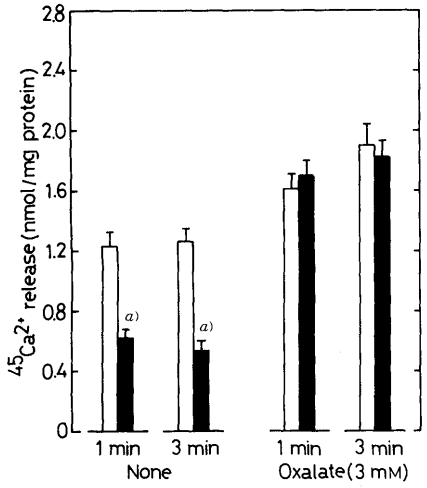

Fig. 10. Effect of Oxalate on Regucalcin-Induced Release of $^{45}Ca^{2+}$ from the Microsomes of Rat Liver

$^{45}Ca^{2+}$ release was measured as described in the experimental section. The microsomes were incubated in the presence of 3 mM oxalate for 5 min after the addition of ATP, and then regucalcin was added to give a final concentration of 2.0 μM. The microsomes were incubated for 1 or 3 min after the addition of regucalcin. Each value represents the mean ± S.E.M. of 5 experiments. *a)* $p<0.01$, as compared with the value without regucalcin. □, none; ■, regucalcin.

Discussion

It has been recognized that liver microsomes have an energy-dependent Ca^{2+} sequestration activity.[15,20-22] Ca^{2+} uptake by liver microsomes may be hormone-sensitive.[21,23] Such a function of liver microsomes plays a role in the regulation of the levels of cytosolic Ca^{2+} in the mammalian liver cells.[20-23] The present investigation was undertaken to clarify whether regucalcin, a calcium-binding protein in hepatic cytosol,[6] influences Ca^{2+} uptake in microsomes isolated from rat liver. The presence of regucalcin (1.0—4.0 μM) in the incubation mixture caused the retardation of $^{45}Ca^{2+}$ uptake by liver microsomes after the addition of ATP. This retardation was seen during 5 min. With longer time, the effect of regucalcin seemed reversible. The effect of regucalcin to retard the microsomal $^{45}Ca^{2+}$ uptake was weakened by the presence of calmodulin, which can stimulate Ca^{2+} uptake by rat liver microsomes.[15] It appears that the effect of regucalcin on the microsomal Ca^{2+} uptake is not an important role in the regulatory system of Ca^{2+} in liver cells. Moreover, the presence of oxalate, a Ca^{2+}-trapping agent, in the incubation mixture, completely blocked the effect of regucalcin to retard $^{45}Ca^{2+}$ uptake by the microsomes. This result is consistent with the view that regucalcin-induced retardation of Ca^{2+} uptake by liver microsomes is dependent on Ca^{2+} release from the microsomes.

When liver microsomes were incubated for 5 min after the addition of ATP, the microsomal $^{45}Ca^{2+}$ uptake was saturated. It was investigated whether regucalcin can stimulate $^{45}Ca^{2+}$ release from microsomes which have accumulated the metal sufficiently. The microsomal $^{45}Ca^{2+}$ was rapidly released during 0.5 min after the addition of regucalcin. The $^{45}Ca^{2+}$ release was seen at 0.5 μM regucalcin, whereas the retardative effect of regucalcin on the microsomal $^{45}Ca^{2+}$ uptake was not caused by this concentration of regucalcin. Thus, regucalcin has a more sensitive effect on Ca^{2+} release than Ca^{2+} uptake. The stimulatory effect of regucalcin on $^{45}Ca^{2+}$ release from the microsomes was similar to the effect of GTP, which can stimulate Ca^{2+}

release from rat liver microsomes.[12] Moreover, regucalcin could additively enhance the effect of GTP on the microsomal $^{45}Ca^{2+}$ release. From these results, it is suggested that regucalcin plays a cell physiological role in the stimulation of Ca^{2+} release from liver microsomes, and that the mechanism of regucalcin action differs from that of GTP.

It has been reported that microsomal Ca^{2+} sequestration is critically dependent on protein sulfhydryl groups, and that modification of protein thiols may be an important mechanism for the inhibition of microsomal Ca^{2+} sequestration by a variety of toxic agents.[24] The ability of regucalcin to stimulate $^{45}Ca^{2+}$ release from liver microsomes was examined in the presence of dithiothreitol, a sulfhydryl group-protecting agent. Regucalcin could stimulate $^{45}Ca^{2+}$ release from the microsomes in the presence of dithiothreitol. This indicates that regucalcin does not act on protein sulfhydryl groups of microsomes to stimulate $^{45}Ca^{2+}$ release.

The stimulatory effect of regucalcin on $^{45}Ca^{2+}$ release from liver microsomes was completely blocked by the presence of oxalate, a Ca^{2+}-trapping agent. Presumably, regucalcin does not directly bind Ca^{2+} in the microsomes, although the protein has a high-affinity binding activity for Ca^{2+}.[6] Since regucalcin can bind to the microsomes of rat liver, it is a possibility that the protein affects membrane components related to the Ca^{2+}-releasing mechanism in the microsomes. Recently, it has been proposed that microsomal Ca^{2+} may be released through a Ca^{2+}-release channel.[25,26] It is possible that regucalcin acts as a Ca^{2+}-release channel on hepatic microsomal membranes. The Ca^{2+}-releasing mechanism of regucalcin, however, remains to be elucidated.

Recently, it has been demonstrated that inositol 1,4,5-triphosphate, which is a second messenger for hormonal stimulation, can release Ca^{2+} from rat liver microsomes.[12,13] The present finding, that regucalcin can stimulate Ca^{2+} release from liver microsomes and retard Ca^{2+} uptake by the microsomes, suggests that regucalcin plays an important role in the elevation of the cytosolic Ca^{2+} level in liver cells. However, regucalcin can bind Ca^{2+}; the value of K_d is 2.4×10^{-6} M. Regucalcin may bind Ca^{2+} when the cytosolic Ca^{2+} concentration increases over 2.4 μM. The concentration of Ca^{2+} in the cytosol of resting liver cells is about 0.1 μM. Therefore, regucalcin may stimulate Ca^{2+} release from the microsomes to regulate liver cell function related to Ca^{2+}, and the protein may bind Ca^{2+} when the cytosolic Ca^{2+} concentration rises over 2.4 μM. Thus, regucalcin may regulate the cytosolic Ca^{2+} concentration in liver cells through dual actions.

In conclusion, it has been demonstrated that regucalcin can stimulate Ca^{2+} release from rat liver microsomes and retard Ca^{2+} uptake by the microsomes. The present finding clearly indicates that regucalcin regulates the Ca^{2+} transport system in liver microsomes. This further supports the view that regucalcin plays a cell physiological role as a regulatory protein for Ca^{2+} action in liver cells.

Acknowledgement This work was supported in part by a Grant-in-Aid (No. 63571053) from the Ministry of Education, Science and Culture, Japan.

References

1) J. R. Williamson, R. H. Cooper, and J. B. Hoek, *Biochim. Biophys. Acta*, **639**, 243 (1981).
2) P. H. Reinhart, W. N. Taylor, and F. L. Bygrave, *Biochem. J.*, **223**, 1 (1984).
3) W. Y. Cheung, *Science*, **207**, 19 (1980).
4) A. R. Means and J. R. Dedman, *Nature* (London), **285**, 73 (1980).
5) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **26**, 1915 (1978).
6) M. Yamaguchi and K. Sugii, *Chem. Pharm. Bull.*, **29**, 567 (1981).
7) M. Yamaguchi and H. Yoshida, *Chem. Pharm. Bull.*, **33**, 4489 (1985).
8) M. Yamaguchi and H. Shibano, *Chem. Pharm. Bull.*, **35**, 2025 (1987).
9) M. Yamaguchi and H. Shibano, *Chem. Pharm. Bull.*, **35**, 2581 (1987).
10) M. Yamaguchi and S. Mori, *Chem. Pharm. Bull.*, **36**, 321 (1988).
11) M. Yamaguchi, S. Mori, and S. Kato, *Chem. Pharm. Bull.*, **36**, 3532 (1988).
12) A. P. Dawson and R. F. Irvine, *Biochem. Biophys. Res. Commun.*, **120**, 858 (1984).
13) S. K. Joseph and J. R. Williamson, *J. Biol. Chem.*, **261**, 14658 (1986).
14) C. R. Fleschner and N. Kraus-Friedmann, *Eur. J. Biochem.*, **154**, 313 (1986).
15) P. B. Moore and N. Kraus-Friedmann, *Biochem. J.*, **214**, 69 (1983).
16) L. Moore, *Life Sci.*, **32**, 741 (1983).
17) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
18) C. V. Nicchitta, S. K. Joseph, and J. R. Williamson, *Biochem. J.*, **248**, 741 (1987).
19) J. G. Comerford and A. P. Dawson, *Biochem. J.*, **249**, 89 (1988).
20) L. Moore, T. Chen, H. R. Knapp, Jr., and E. J. Landon, *J. Biol. Chem.*, **250**, 4562 (1975).
21) N. Friedmann and F. D. Johnson, *Life Sci.*, **27**, 837 (1980).
22) C. Heilmann, C. Spamer, and W. Gerok, *J. Biol. Chem.*, **260**, 788 (1985).
23) A-M. A. Waltenbaugh and N. Friedmann, *Biochem. Biophys. Res. Commun.*, **82**, 603 (1978).
24) H. Thor, P. Hartzell, S-A. Svensson, S. Orrenius, F. Mirabelli, V. Marimoni, and G. Bellomo, *Biochem. Pharmacol.*, **34**, 3717 (1985).
25) A. Yatani, J. Codina, A. M. Brown, and L. Birnbaumer, *Science*, **235**, 207 (1986).
26) J. Codina, S. Kimura, and N. Kraus-Friedmann, *Biochem. Biophys. Res. Commun.*, **150**, 848 (1988).