Regucalcin-Induced Ca^{2+} Release from Rat Liver Microsomes: The Effect Is Inhibited by Heparin

Masayoshi YAMAGUCHI* and Seiichi MORI

Department of Environmental Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 395 Yada, Shizuoka City 422, Japan.
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The effect of heparin on the calcium-binding protein regucalcin-stimulated Ca^{2+} release from rat liver microsomes was investigated. Ca^{2+} release was assayed by the method of Millipore filtration to estimate microsomal $^{45}Ca^{2+}$ accumulation following the addition of 10 mM adenosine triphosphate. The addition of regucalcin (1.0 μM) or inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3; 1.0 μM] stimulated $^{45}Ca^{2+}$ release from rat liver microsomes. These effects were completely inhibited by the presence of heparin (10.0 μg/ml). Regucalcin did not enhance the effect of Ins(1,4,5)P_3. These results suggest that regucalcin affects $^{45}Ca^{2+}$ release involved in Ins(1,4,5)P_3 action in rat liver microsomes.

Keywords calcium; regucalcin; inositol 1,4,5-trisphosphate; heparin; rat liver microsome

In recent years, we have reported that a calcium-binding protein (regucalcin), which differs from calmodulin, is distibuted in the hepatic cytosol of rats.[1,2] 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.[2] This novel protein has a reversible effect on the activation of various enzymes by Ca^{2+} in liver cells.[3-5] It is proposed that regucalcin may play a role as a regulatory protein for Ca^{2+} in liver cells, although the physiological role of regucalcin has not yet been clarified fully.

On the other hand, inositol 1,4,5-trisphosphate [Ins-(1,4,5)P_3], which is a second messenger for hormonal stimulation, can release Ca^{2+} from rat liver microsomes.[6,7] It has previously been shown that the sulphated polysaccharide, heparin, can compete with Ins(1,4,5)P_3 for its specific binding site.[8,9] More recently, we have reported that regucalcin stimulates Ca^{2+} release from rat liver microsomes.[10] Therefore, the present study was undertaken to clarify whether or not the regucalcin-induced Ca^{2+} release from the microsomes is inhibited by heparin. It was found that heparin completely inhibited the regucalcin-promoted Ca^{2+} release.

Materials and Methods

$^{45}CaCl_2$ (specific activity 12.4 GBq/mg) used was obtained from New England Nuclear (Boston, Mass., U.S.A.). Heparin (sodium salt, grade 1) and D-myo-inositol 1,4,5-trisphosphate (from bovine brain) were purchased from the Sigma Chemical Co., (St. Louis, Mo., U.S.A.). Other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan).

Male Wistar rats, weighing 100—120 g, were obtained commercially from the Japan SLC, Inc. (Hamamatsu, Japan). Rats were killed by cardiac puncture and the livers were 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 (EDTA) and 1 mM dithiothreitol, pH 7.2 and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle.[11] The homogenate was centrifuged at $1000 \times g$ for 10 min. 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 was measured by the Millipore filtration technique.[12,13] 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. The incubation mixture was further incubated for 5 min after the addition of 10 mM adenosine triphosphate (ATP; adjusted to pH 6.8 with KOH) to initiate energy-dependent Ca^{2+} uptake, and then regucalcin (1 μM) or Ins(1,4,6)P_3 (1 μM) was added to the incubation mixture. At a designated time after the addition of regucalcin or Ins(1,4,5)P_3, 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 vesicle. The precipitate on the 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. Protein was determined by the method of Lowry *et al.*,[14] using bovine serum albumin as the standard.

Regucalcin in the cytosol fraction ($105000 \times g$ supernatant) of rat liver homogenate 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 previously reported.[1]

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

Isolated liver microsomes were incubated for 1 min in a medium containing $^{45}Ca^{2+}$, and then 10 mM ATP was added and further incubated for 5 min. The microsomal $^{45}Ca^{2+}$ uptake was saturated by incubation for 5 min with 10 mM ATP. At this time, regucalcin (1 μM) and/or Ins(1,4,5)P_3 was added to the incubation mixture. The addition of regucalcin caused a rapid release of $^{45}Ca^{2+}$ from the

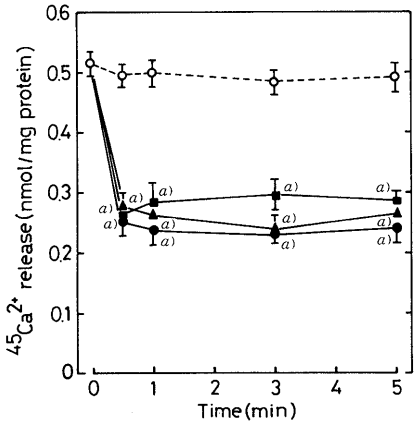

Fig. 1. Effect of Regucalcin and/or Ins(1,4,5)P_3 on $^{45}Ca^{2+}$ Release from the Microsomes of Rat Liver

$^{45}Ca^{2+}$ 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 10 mM ATP. At zero time regucalcin and/or Ins(1,4,5)P_3 was added to give a final concentration of 1.0 μM. Each value represents the mean ± S.E.M. of five experiments. *a)* $p < 0.01$, as compared with the value without regucalcin and/or Ins(1,4,5)P_3. ○, control; ●, regucalcin; ■, Ins(1,4,5)P_3; ▲, regucalcin plus Ins(1,4,5)P_3.

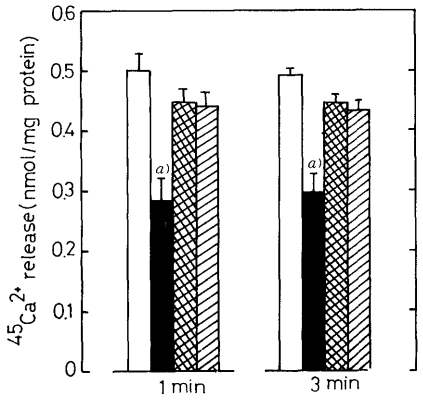

Fig. 2. Effect of Heparin on Ins(1,4,5)P_3-Induced Release of $^{45}Ca^{2+}$ from Rat Liver Microsomes

The microsomes were incubated for 5 min after the addition of 10 mM ATP, and then Ins(1,4,5)P_3 was added to give a final concentration of 1.0 μM immediately after the addition of heparin (10 μg/ml). The microsomes were incubated for 1 or 3 min after the addition of Ins(1,4,5)P_3. Each value represents the mean ± S.E.M. of five experiments. *a)* $p<0.01$, as compared with the value of control. □, control; ■, Ins(1,4,5)P_3; ▩, Ins(1,4,5)P_3 plus heparin; ▨, heparin.

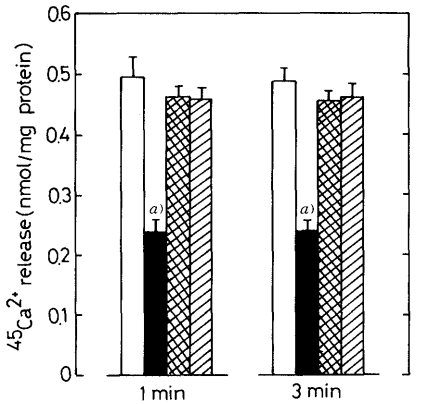

Fig. 3. Effect of Heparin on Regucalcin-Induced Release of $^{45}Ca^{2+}$ from Rat Liver Microsomes

The microsomes were incubated for 5 min after the addition of 10 mM ATP, and then regucalcin was added to give a final concentration of 1.0 μM immediately after the addition of heparin (10 μg/ml). The microsomes were incubated for 1 or 3 min after the addition of regucalcin. Each value represents the mean ± S.E.M. of five experiments. *a)* $p<0.01$, as compared with the value of control. □, control; ■, regucalcin; ▩, regucalcin plus heparin; ▨, heparin.

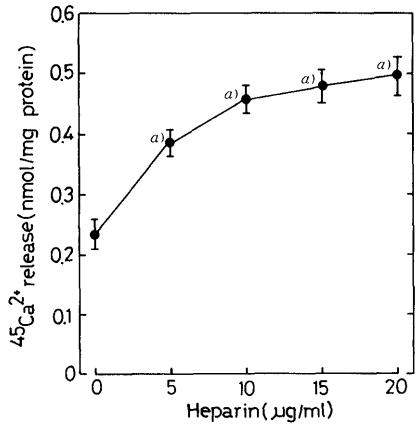

Fig. 4. Effect of Increasing Concentrations of Heparin on $^{45}Ca^{2+}$ Release from Rat Liver Microsomes

The microsomes were incubated for 5 min after the addition of 10 mM ATP, and then regucalcin was added to give a final concentration of 1.0 μM immediately after the addition of heparin (5, 10, 15, and 20 μg/ml). The microsomes were incubated for 1 min after the addition of regucalcin. Each value represents the mean ± S.E.M. of five experiments. *a)* $p<0.01$, as compared with the value without heparin.

microsomes during 0.5 min (Fig. 1). The amount of $^{45}Ca^{2+}$ released by regucalcin was about 50% of $^{45}Ca^{2+}$ accumulated in the microsomes. The regucalcin-induced $^{45}Ca^{2+}$ release from the microsomes was not significantly enhanced by the presence of Ins(1,4,5)P_3 (1 μM), although the sugar caused microsomal $^{45}Ca^{2+}$ release.

The effect of heparin on Ins(1,4,5)P_3-induced release of $^{45}Ca^{2+}$ from the microsomes is shown in Fig. 2. The presence of heparin (10 μg/ml) caused a complete inhibition of the microsomal $^{45}Ca^{2+}$ release induced by 1 μM Ins(1,4,5)P_3. Heparin itself did not have an appreciable modification of the microsomal $^{45}Ca^{2+}$ release.

Also, the regucalcin (1 μM)-induced $^{45}Ca^{2+}$ release from liver microsomes was completely inhibited by the presence of heparin (10 μg/ml) (Fig. 3). The effect of increasing concentrations of heparin (5.0, 10, 15, and 20 μg/ml) on regucalcin (1 μM)-induced release of $^{45}Ca^{2+}$ from the microsomes is shown in Fig. 4. The results indicated that regucalcin-stimulated $^{45}Ca^{2+}$ release is inhibited by low concentrations of heparin. A half-maximal effect was obtained at approximately 5.0 μg/ml of heparin.

Discussion

Liver microsomes have an energy-dependent Ca^{2+} sequestration activity.[12,15,16] When liver microsomes were incubated for 5 min after the addition of ATP, the microsomal $^{45}Ca^{2+}$ uptake was saturated. Regucalcin could 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 previous investigation showed that the mechanism of regucalcin action on hepatic microsomal $^{45}Ca^{2+}$ release differed from that of guanosine triphosphate (GTP), and that regucalcin did not act on protein sulfhydryl groups of microsomes to stimulate $^{45}Ca^{2+}$ release.[10]

Heparin could fairly inhibit the Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release from liver microsomes. This effect of heparin may be based on the competition with Ins(1,4,5)P_3 for its specific binding site.[8,9] Also, the stimulatory effect of regucalcin on microsomal $^{45}Ca^{2+}$ release was completely inhibited by heparin. From this result, it is possible that regucalcin may have an affect on the specific binding site for Ins(1,4,5)P_3, and the protein may stimulate Ca^{2+} release from liver microsomes. Regucalcin could not enhance the effect of Ins(1,4,5)P_3 on microsomal $^{45}Ca^{2+}$ release, although the protein additively enhanced the GTP-induced Ca^{2+} release from the microsomes.[10] This may support the view that regucalcin affects the binding site of Ins(1,4,5)P_3 in liver microsomes, and that the mode of regucalcin action differs from that of GTP. We have reported that radioiodinated regucalcin can bind to liver microsomes.[17] Whether or not regucalcin binds to the specific binding component (receptors) of Ins(1,4,5)P_3 on liver microsomes has not yet been clarified. Since there are specific binding components of regucalcin in liver microsomes, the components may locate close to the specific binding sites of Ins(1,4,5)P_3.

In conclusion, hepatic calcium-binding protein regucalcin can promote microsomal Ca^{2+} release, and the mechanism may involve that of Ins(1,4,5)P_3 action. A physiological significance of regucalcin in Ca^{2+} homeostasis, however, is unknown.

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