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Suppressive Role of Endogenous Regucalcin in the Enhancement of Protein Kinase Activity With Proliferation of Cloned Rat Hepatoma Cells (H4-II-E)

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Abstract The role of endogenous regucalcin, which is a regulatory protein in calcium signaling, in the regulation of protein kinase activity in the proliferation of the cloned rat hepatoma cells (H4-II-E) was investigated. Hepatoma cells were cultured for 6–72 h in the presence of fetal bovine serum (FBS; 1 or 10%). The number of cells and protein kinase activity in the 5500 g supernatant of cell homogenate was significantly increased 24 and 48 h after the culture with FBS (1 or 10%); the culture with 10% FBS was potent effect as compared with that of 1% FBS. FBS (10%)-increased protein kinase activity preceded a significant elevation of cell number of 6 h after culture. Serum stimulation-induced increase in protein kinase activity was significantly decreased in the presence of trifluoperazine (50 μM), staurosporine (10^{-6} M) or genistein (10^{-5} M) in the enzyme reaction mixture. The presence of anti-regucalcin monoclonal antibody (40 or 80 ng/ml) in the reaction mixture caused a significant increase in protein kinase activity in the cells cultured with FBS (1 or 10%). This increase was completely blocked by addition of regucalcin (10^{-6} M), which can reveal an inhibitory effect on protein kinase activity. Moreover, the effect of antibody in increasing protein kinase activity was significantly inhibited in the presence of trifluoperazine, staurosporine, or genistein, indicating that endogenous regucalcin has an inhibitory effect on Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, and protein tyrosine kinase. The present study suggests that endogenous regucalcin plays a suppressive role in the enhancement of various protein kinase activities associated with a proliferation of the cloned rat hepatoma cells (H4-II-E). J. Cell. Biochem. Suppl. 36:12–18, 2001.

Key words: regucalcin; protein kinase; calcium signaling; cell proliferation; rat hepatoma cells

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. The Ca^{2+} effect in cells is amplified by calmodulin and protein kinase C, which are related to a signal transduction due to hormonal stimulation [Cheung, 1980; Nishizuka, 1986; Heizman and Hunziker, 1991; Kraus-Friedman and Feng, 1996]. Regucalcin, which was found as a novel Ca^{2+}-binding protein [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1988], has been demonstrated to play a multifunctional role as a regulatory protein in the Ca^{2+}-signaling process [Yamaguchi, 2000a, b] in recent years.

The regucalcin gene is localized on rat chromosome Xq 11.1–12 proximal end, and regucalcin messenger ribonucleic acid (mRNA) and its protein are greately present in liver and kidney cortex [Shimokawa and Yamaguchi, 1993a, 1993b; Yamaguchi and Isogai, 1993; Shimokawa et al., 1995]. The expression of liver regucalcin mRNA is mediated through Ca^{2+}-signaling mechanism [Shimokawa and Yamaguchi, 1993a; Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999]. Regucalcin has been shown to inhibit Ca^{2+} signaling in liver and kidney cortex cells, and the mRNA expression is stimulated by Ca^{2+}-dependent process due to hormone stimulation [Yamaguchi, 2000a,b].

Regucalcin, more recently, has been shown to regulate liver nuclear function; it can inhibit deoxyribonucleic acid (DNA) and RNA synthesis in normal liver and regenerating rat liver [Yamaguchi and Kanayama, 1996; Yamaguchi

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and Ueoka, 1997]. Regucalcin has also inhibitory effect on protein kinase and protein phosphatase activities in the nucleus of regenerating rat liver [Katsumata and Yamaguchi, 1998; Omura and Yamaguchi, 1999]. Regucalcin may play a regulatory role in proliferative liver cells. This, however, has not fully clarified in cell culture system.

The expression of regucalcin mRNA and its protein has been demonstrated in the cloned rat hepatoma cells (H4-II-E), and the expression is stimulated by Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1999; Inagaki et al., 2000]. This cell may be a tool to study a role of regucalcin in the regulation of cell proliferation. Regucalcin has been shown to inhibit protein tyrosine phosphatase in the cloned rat hepatoma cells (H4-II-E) cultured with fetal bovine serum (FBS) [Inagaki et al., 2000; Inagaki and Yamaguchi, 2000]. The present study, moreover, was undertaken to clarify the effect of regucalcin on protein kinase activity in the proliferation of cloned rat hepatoma cells (H4-II-E) stimulated by FBS. We found that an increase in protein kinase activity due to serum stimulation precedes cell proliferation, and endogenous regucalcin plays a suppressive role in the enhancement of the enzyme activity with cell proliferation.

MATERIALS AND METHODS

Chemicals

α-Minimum essential medium (α-MEM) and penicillin-streptomycin solution (5000 U/ml penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), calmodulin [52000 U/mg protein from bovine], 1,2-dioctanoyl-rac-glycerol (DG; from bovine brain), adenosine triphosphate (ATP), trifluoperazine, staurosporine, genistein, and monimmune IgG were obtained from Sigma Chemicals Co. (St. Louis, MO). [γ-^{32}P]ATP(111 TBq/mmol) was purchased from New England Nuclear (Boston, MA). NHS-LC-biotin was obtained from Pierce (Rockford, IL). Streptavidin-peroxidase conjugate was obtained from Tago Inc. (Burlingame, CA). Calcium chloride and other chemicals were purchased from Sigma Chemical Co. and Wako Pure Chemical Company (Osaka, Japan). The reagents were dissolved in distilled water or ethanol solution. Some reagents were passed through ion-exchange resin to remove metal ions.

Animals

Male Wistar rats, weighing 100–120 *g*, were used. They were obtained commercially from the 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 is markedly expressed in rat liver cytosol [Yamaguchi and Isogai, 1993]. Regucalcin was isolated from rat liver cytosol. The livers were perfused with Tris-HCl buffer (pH 7.4), containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4°C. The livers were removed, cut into small pieces, suspended 1:4 (weight: volume) in Tris-HCl buffer (pH 7.4); the homogenate was spun at 5,500 *g* in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000 *g* for 60 min. The resulting supernatant was heated at 60°C for 10 min and recentrifuged at 38,000 *g* for 20 min. Regucalcin in the supernatant 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 [Yamaguchi and Yamamoto, 1978].

Anti-Regucalcin Antibody

Anti-regucalcin antibody was a monoclonal antibody raised against regucalcin prepared using standard methods [Omura and Yamaguchi, 1999]. Mice (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg per animal of antigen (rat liver regucalcin) emulsified with Freund's complete adjuvant, and 19 d later antigen (0.25 mg/animal) was intraperitoneally injected with Freund's incomplete adjuvant. Animals were killed by bleeding 3 d after the last injection. Spleen cells were prepared from immunized mouse and fused into myeloma cells. Anti-regucalcin monoclonal antibody-producing cells (hybridoma cells) were obtained by screening. The IgG from hybridoma cells was isolated through a protein A-agarose column (Sigma).

Cell Culture

Generation of H4-II-E hepatoma cells (1×10^6) were maintained for 6–72 h in α-MEM supplemented with 5 mM glucose, 1 or 10% heat-inactivated fetal bovine serum (FBS),

50 U/ml penicillin, and 50 μg/ml streptomycin in humidified 5% CO_2/95% air at 37°C to obtain confluent monolayers [Yamaguchi and Nakajima, 1999]. After culture, cells were washed three times with PBS, scraped into 0.5 ml of ice-cold 0.25 M sucrose solution containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml leupeptin, and disrupted for 60 s with an ultrasonic device. Scraped cells were also homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were spun at 5,500 *g* a refrigerated centrifuge for 10 min to obtain cytosol. The 5,000 *g* supernatant fraction was used to assay protein kinase activity. Protein concentration in the 5,500 *g* supernatant fraction was determined by the method of Lowry et al. [1951] using bovine serum albumin as the standard.

Assay of Protein Kinase Activity

Protein kinase activity was measured at 30°C for 10 min in an incubation volume of 500 μl as described by Connelly et al. [1987]. Phosphorylation of the substrate was performed in a reaction mixture containing the 5,500 *g* supernatant of cell homogenate (300–500 μg protein/ml), 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.2 mM EGTA, 20 μM ATP, 5.55 TBq [γ-^{32}P] ATP in the presence or absence of 0.5 mM $CaCl_2$. In separate experiments, the enzyme reaction mixture contained either vehicle, TFP (50 μM), staurosporine (10^{-6} M), genistein (10^{-5} M), calmodulin (10 μg/ml), DG (10 μg/ml), regucalcin (10^{-8} or 10^{-7} M) or anti-regucalcin monoclonal antibody (20–80 ng/ml) without or with Ca^{2+} addition. The phosphorylation reaction was terminated by the addition of 2 ml of ice-cold 5% trichloroacetic acid containing 10 mM H_3PO_4. The radioactivity retained on GF/B glass fiber filters after filtration was determined by counting the dried filters in 2 ml of scintillation fluid. Protein kinase activity was determined after substracting the incorporation in the presence of 1 mM ethylene glycol bis(2-amino-ethylether) N,N, N′,N′-tetraacetic acid (EGTA, pH 7.0) without Ca^{2+} addition. The enzyme activity was expressed as the radioactivity (dpm) of [^{32}P]phosphate phosphorylated per milligram of the nuclear protein during incubation (10 min).

Statistical Analysis

Data were expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. A *P* value of less than 0.05 was considered to indicate a statistically significant difference.

RESUTLS

Enhancement of Protein Kinase Activity in Proliferative Cells

The cloned rat hepatoma cells (H4-II-E) were cultured for 6, 24, 48, and 72 h in the presence of FBS (1 or 10%). The alteration in cell number and protein kinase activity in the 5500 *g* supernatant of cell homogenate is shown in Figure 1. The number of cells was markedly increased in the presence of 10% FBS (Fig. 1A). A significant increase in cell number was seen at 24 h after culture. Such an increase was not observed by the culture with 1% FBS. Cell number, however, was significantly increased 48 h after the culture with 1% FBS. Meanwhile, protein kinase activity in the 5,500 *g* supernatant of cell homogenate was significantly increased between 6 and 72 h after the culture; the maximum increase was seen at 24 and 48 h after culture (Fig. 1B). The cell culture with 1% FBS caused a significant increase in the enzyme activity at 24 and 48 h after the culture. An increase in protein kinase activity in the cell was preceded to an elevation of cell number in the presence of FBS (1 or 10%).

The characterization of protein kinase activity in the cloned rat hepatoma cells was examined. The cloned rat hepatoma cells was cultured for 6, 24, 48, and 72 h in the presence of

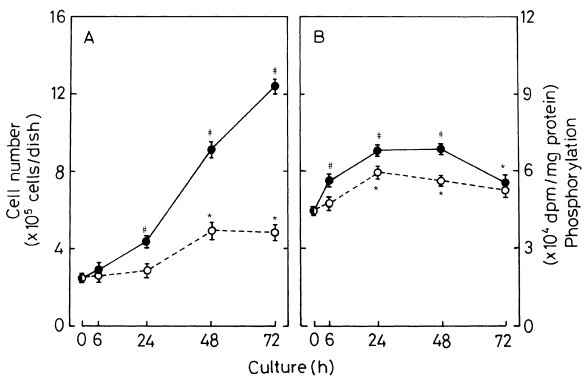

Fig. 1. Increase in cell numbers **(A)** and protein kinase activity **(B)** of the cloned rat hepatoma cells (H3-II-E) cultured with different concentrations of FBS. Cells (2.5×10^5) were cultured 6, 24, 48, and 72 h in the presence of FBS (1 or 10%). Each value is the mean ± SEM of five experiments. $^*P < 0.01$, compared with the value obtained from zero time. $^{\#}P < 0.01$, compared with the value obtained by culture with 1% FBS. Open circles, 1% FBS; closed circles, 10% FBS.

FBS (1 or 10%). After culture, protein kinase activity in the 5500 *g* supernatant fraction of cell homogenate was significantly raised in the presence of calmodulin (10 μg/ml) or dioctanoylglycerol (10 μg/ml) with calcium chloride addition (0.5 mM) (Figs. 2,3). This result indicates that Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C are present in the cloned rat hepatoma cells.

The effect of various protein kinase inhibitors on protein kinase activity in the cloned rat hepatoma cells (H4-II-E) is shown in Figure 4. Cells were cultured for 24 or 48 h in the presence of FBS (1 or 10%). Protein kinase activity in the 5500 *g* supernatant of cell homogenate obtained by 24 or 48 h culture was significantly decreased in the presence of trifluoperazine (50 μM), an antagonist of Ca^{2+}/calmodulin-dependent protein kinase [Vincenzi, 1982]. Staurosporine (10^{-6} M), an inhibitor of protein kinase C [Tamaoki et al., 1986], or genistein (10^{-5} M), an inhibitor of protein tyrosine kinase [Liu et al., 1994]. The effect of various inhibitors in decreasing protein kinases might further be enmhanced using higher concentrations.

Suppressive Effect of Endogenous Regucalcin in the Enhancement of Protein Kinase Activity in Proliferative Cells

The effect of regucalcin on protein kinase activity in the cloned rat hepatoma cells (H4-II-E) is shown in Figure 5. Cells were cultured for 24 or 48 h in the presence of FBS (1 or 10%), and the 5500 *g* supernatant of cell homogenate was prepared. The addition of regucalcin (10^{-8} or 10^{-7} M) in the enzyme reaction mixture caused a significant decrease in protein kinase activity with calcium chloride (0.5 mM) addition. The effect of regucalcin in decreasing protein kinase activity was seen in the cells cultured with 1 or 10% FBS.

The effect of anti-regucalcin monoclonal antibody on protein kinase activity in the cloned rat hepatoma cells (H4-II-E) is shown in Figure 6. Cells were cultured for 24 or 48 h in the presence of FBS (1 or 10%). Protein kinase activity in the 5500 *g* supernatant of cell homogenate of hepatoma cells cultured with 10% FBS was significantly increased in the presence of anti-regucalcin monoclonal antibody (20 ng/ml) in increasing the enzyme activity was not seen in the cells cultured with 1% FBS, although the higher concentrations (40 or 80 ng/ml) of antibody had a significant effect on the enzyme activity. The anti-regucalcin monoclonal antibody (80 ng/ml)-increased protein kinase activity in the 5500 *g* supernatant of cell homogenate obtained at 24 or 48 h after the culture with FBS (1 or 10%) was completely abolished by the addition of regucalcin (10^{-6} M) (data not shown). Meanwhile, the presence of nonimmune IgG (100 ng/ml) in the enzyme reaction mixture did

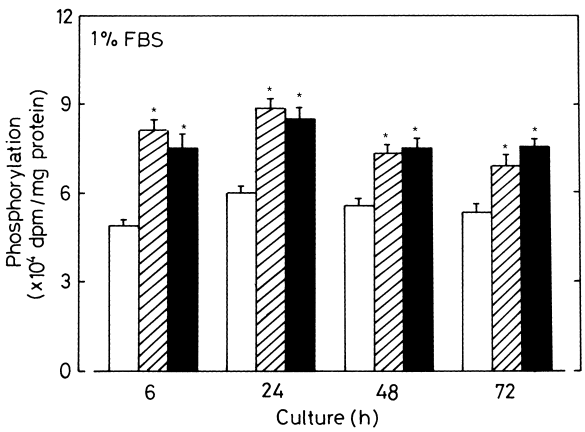

Fig. 2. Effect of calmodulin or dioctanoylglycerol on protein kinase activity in the cloned rat hetapoma cells (H4-II-E) cultured with 1% FBS. Cells were cultured for 6, 24, 48, and 72 h in the presence of FBS (1%). The enzyme reaction mixture contained either vehicle, calmodulin (10 μg/ml) or dioctanoylglycerol (10 μg/ml) with calcium chloride (0.5 mM) addition. Each value is the mean ± SEM of five experiments. $^*P < 0.01$, compared with the control (none) value. White bars, control; hatched bars, calmodulin; black bars, dioctanoylglycerol.

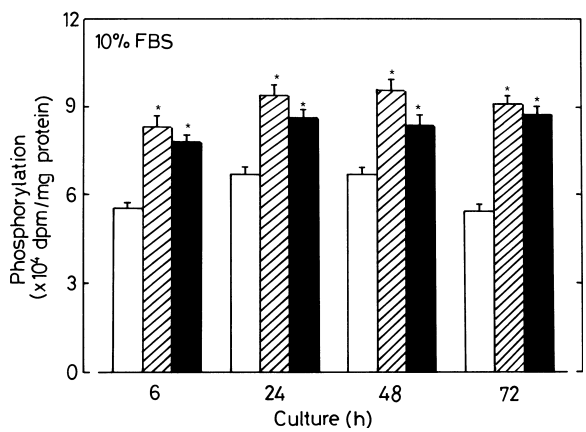

Fig. 3. Effect of calmodulin or dioctanoylglycerol on protein kinase activity in the cloned rat hepatoma cells (H4-II-E) cultured with 10% FBS. Cells were cultured for 6, 24, 48, and 72 h in the presence of FBS (10%). The enzyme reaction mixture contained either vehicle, calmodulin (10μg/ml) or dioctanoylglycerol (10 μg/ml) with calcium chloride (0.5 mM) addition. Each value is the mean ± SEM of five experiments. $^*P < 0.01$, compared with the control (none) value. White bars, control; hatched bars, calmodulin; black bars, dioctanoylglycerol.

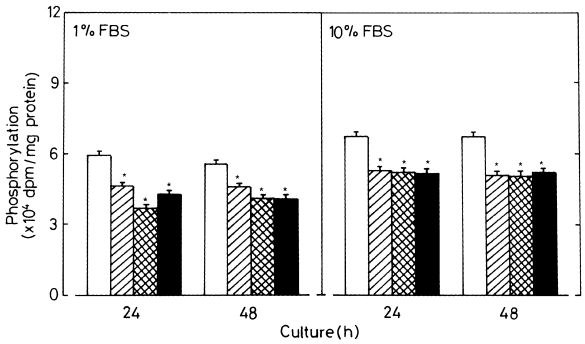

Fig. 4. Effect of various inhibitors on protein kinase activity in the cloned rat hepatoma cells (H4-II-E). Cells were cultured for 24 or 48 h in the presence of FBS (1 or 10%). The enzyme reaction mixture contained either vehicle, trifluoperazine (50 μM), staurosporine (10^{-6} M) or genistein (10^{-5} M) with calcium chloride (0.5 mM) addition. Each value is the mean ± SEM of five experiments. *$P < 0.01$, compared with the control (none) value. White bars, control; hatched bars, trifluoperazine; double hatched bars, staurosporine; black bars, genistein.

not have a significant effect on protein kinase activity in the cells cultured for 24 or 48 h with FBS (1 or 10%) (data not shown).

The effect of various protein kinase inhibitors on the anti-regucalcin monoclonal antibody-increased protein kinase activity in the cloned rat hepatoma cells (H4-II-E) is shown in Figure 7. The effect of anti-regucalcin monoclonal antibody (80 ng/ml) in increasing the enzyme activity in the cells obtained by 24 or 48 h-culture with FBS (1 or 10%) was significantly inhibited in the presence of trifluoperazine

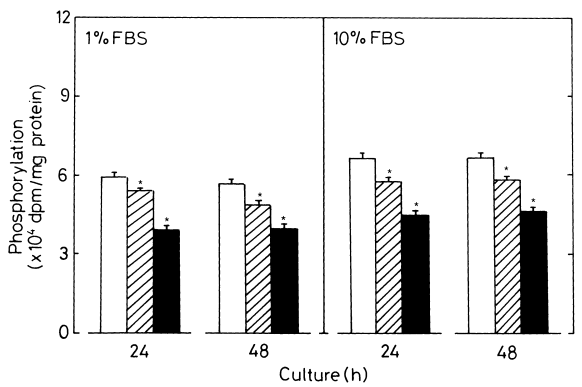

Fig. 5. Effect of regucalcin on protein kinase activity in the cloned rat hepatoma cells (H4-II-E). Cells were cultured for 24 or 48 h in the presence of FBS (1 or 10%). The enzyme reaction mixture contained either vehicle or regucalcin (10^{-7} M) with calmodulin chloride (0.5 mM) addition. Each value is the mean ± SEM of five experiments. *$P < 0.01$, compared with the control (none) value. White bars, control; hatched bars, regucalcin (10^{-8} M); black bars, regucalcin (10^{-7} M).

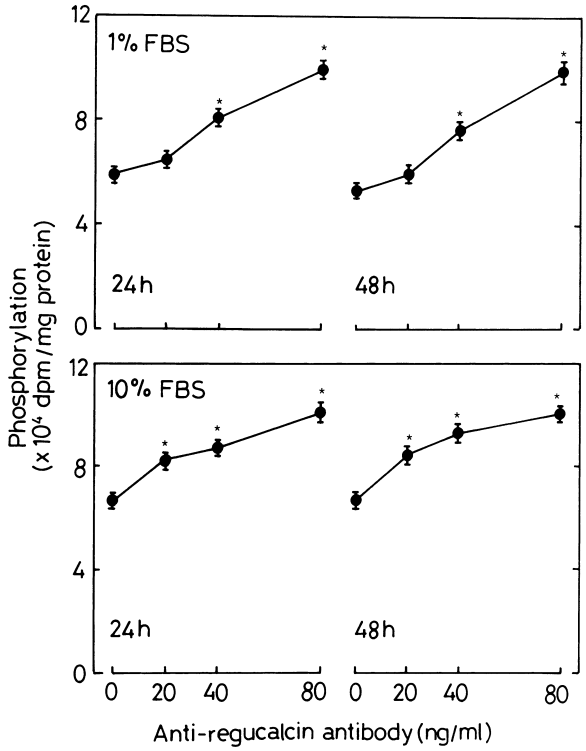

Fig. 6. Effect of anti-regucalcin monoclonal antibody on protein kinase activity in the cloned rat hepatoma cells (H4-II-E). Cells were cultured for 24 or 48 h in the presence of FBS (1 or 10%). The enzyme reaction mixture contained either vehicle or anti-regucalcin antibody (20, 40 or 80 ng/ml) with calcium chloride (0.5 mM) addition. Each value is the mean ± SEM of five experiments. *$P < 0.01$, compared with the control (none) value.

(50 μM), staurosporine (10^{-6} M) or genistein (10^{-5} M) in the reaction mixture.

DISCUSSION

Regucalcin has been demonstrated to have an inhibitory effect on DNA and RNA synthesis, protein kinase and protein phosphatase activities in the nucleus of normal liver and regenerating rat liver after partial hepatectomy [Yamaguchi and Kanayama, 1996; Yamaguchi and Ueoka, 1997; Katsumata and Yamaguchi, 1998; Omura and Yamaguchi, 1999], suggesting a regulatory role of regucalcin in nuclear functions of proliferative cells. An involvement of regucalcin in the regulation of cell proliferation, however, remains to be elucidated. Regucalcin mRNA and its protein are expressed in the cloned rat hepatoma cells (H4-II-E) [Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999; Inagaki et al., 2000]. Regucalcin

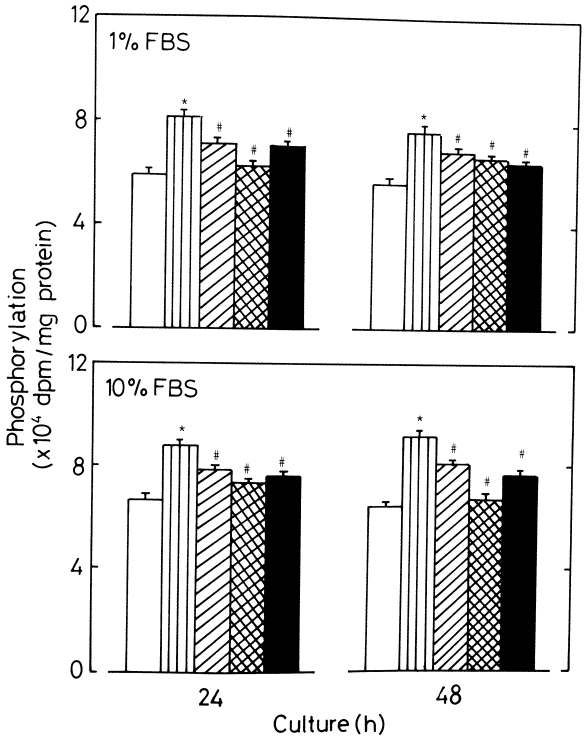

Fig. 7. Effect of protein kinase inhibitors on the anti-regucalcin monoclonal antibody-increased protein kinase activity in the cloned rat hepatoma cells (H4-II-E). Cells were cultured for 24 or 48 h in the presence of FBS (1 or 10%). The enzyme reaction mixture contained either vehicle, trifluoperazine (50 μM), staurosporine (10^{-6} M) or genistein (10^{-5} M) in the absence or presence of anti-regucalcin antibody (40 ng/ml). Each value is the mean ± SEM of five experiments. $^{*}P < 0.01$, compared with the control (non) value. $^{*}P < 0.01$, compared with the value obtained by anti-regucalcin antibody addition. White bars, control; lined bars, anti-regucalcin antibody alone; hatched bars, antibody plus trifluoperazine; double hatched bars, antibody plus staurosporine; black bars, antibody plus genistein.

may play a functional role in the cloned rat hepatoma cells. The present study was undertaken to determine a role of regucalcin in the regulation of proliferation of the cloned hepatoma cells.

The culture with FBS produced an increase in protein kinase activity and a corresponding elevation of cell number in the cloned rat hepatoma cells (H4-II-E). An increase in protein kinase activity of the 5500 *g* supernatant of cell homogenate preceded a significant elevation of cell number, suggesting that serum factors (including growth factors and hormones) stimulate cell proliferation which is partly mediated through cascades of protein kinases. Serum stimulation-elevated protein kinase activity in hepatoma cells was significantly enhanced by the addition of calmodulin or dioctanoylglycerol in the presence of calcium chloride. This result indicates that Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C are present in hepatoma cells. In addition, protein kinase activity in hepatoma cells was significantly inhibited in the presence of trifluoperazine, staurosporine, or genistein in the enzyme reaction mixture. Serum stimulation presumably increases the activities of Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, and protein tyrosine kinase in the cytoplasm of cloned rat hepatoma cells. However, protein kinase activity increased in the cells treated with 1% serum-conditions where cell proliferation was limited. The effect of 10% serum on cell proliferation was greater than that of 1% serum, although the 10% serum effect on protein kinases was not great. This observation suggests that other signaling factors in addition to protein kinases may partly be involved in serum-induced stimulation of cell proliferation.

The presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture containing the 5500 *g* supernatant of cell homogenate of cloned rat hepatoma cells cultured with FBS caused a significant increase in protein kinase activity. This effect was completely abolished by the addition of exogenous regucalcin, which revealed an inhibitory effect on the enzyme activity. This finding indicates that endogenous regucalcin in the cloned rat hepatoma cells suppresses protein kinase activity in the cytoplasm. The anti-regucalcin monoclonal antibody-increased protein kinase activity in the hepatoma cells was significantly inhibited in the presence of trifluoperazine, staurosporine, or genistein, suggesting that endogenous regucalcin inhibits Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, or protein tyrosine kinase activities. Endogenous regucalcin may act various protein kinases in the cytoplasm of cloned rat hepatoma cells.

Serum stimulation may lead to an increase in cell proliferation which is partly mediated through cascade for various protein kinases in the cloned rat hepatoma cells. Regucalcin may have a suppressive effect for overexpression of cell proliferation due to inhibiting various protein kinases in the cytoplasm of cloned hepatoma cells. Whether regucalcin can regulate nuclear functions in the cloned hepatoma cells, moreover, remains to be elucidated. Pre-

sumably, regucalcin plays a regulatory in cell proliferation of normal liver and hepatoma cells.

In conclusion, it has been demonstrated that endogenous regucalcin plays a suppressive role in the enhancement of protein kinase activity associated with proliferation of the cloned rat hepatoma cells (H4-II-E).

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