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Regulatory Role of Endogenous Regucalcin in the Enhancement of Nuclear Deoxyribonuleic Acid Synthesis With Proliferation of Cloned Rat Hepatoma Cells (H4-II-E)

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Abstract The role of endogenous regucalcin in the regulation of deoxyribonuleic acid (DNA) synthesis in the nuclei of the cloned rat hepatoma cells (H4-II-E) with proliferative cells was investigated. Cells were cultured for 6–96 h in a α-minimum essential medium (α-MEM) containing fetal bovine serum (FBS; 1 or 10%). Cell number was significantly increased between 24 and 96 h after culture with 10% FBS; cell proliferation was markedly stimulated by culture with 10% FBS as compared with that of 1% FBS. In vitro DNA synthesis activity in the nuclei of cells was significantly elevated 6 h after culture with 10% FBS and its elevation was remarkable at 12 and 24 h after the culture. Nuclear DNA synthesis activity was significantly reduced in the presence of various protein kinase inhibitors (PD98059, staurosprine, or trifluoperazine) in the reaction mixture containing the nuclei of cells cultured for 12 and 24 h with FBS (1 and 10%). The addition of regucalcin (10^{-7} and 10^{-6}M) in the reaction mixture caused a significant inhibition of nuclear DNA synthesis activity. The presence of anti-regucalcin monoclonal antibody (25–100 ng/ml) in the reaction mixture containing the nuclei of cells cultured for 24 h with 10% FBS resulted in a significant increase in nuclear DNA synthesis activity. This increase was completely blocked by the addition of regucalcin (10^{-6} M). The effect of anti-regucalcin antibody (100 ng/ml) in increasing nuclear DNA synthesis activity was significantly inhibited in the presence of various protein kinase inhibitors. DNA synthesis activity was significantly enhanced in the presence of anti-regucalcin antibody (100 ng/ml) in the reaction mixture containing the nuclei of cells cultured for 24 h with 10% FBS in the presence of Bay K 8644 (2.5×10^{-6} M). Culture with Bay K 8644 did not cause a significant increase in DNA synthesis activity in the absence of anti-regucalcin antibody. The present study demonstrates that endogenous regucalcin plays a suppressive role in the enhancement of nuclear DNA synthesis with proliferative cells. J. Cell. Biochem. 82: 704–711, 2001.

Key words: regucalcin; DNA synthesis; 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 Ca^{2+} -dependent protein kinases, which are related to a signal transduction due to hormonal stimulation [Nishizuka, 1986; Cheung, 1980; 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 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, b; 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; Yamaguchi and Nakajima, 1999; Murata and Yamaguchi, 1999].

Regucalcin has been shown to regulate liver nuclear function; it can inhibit DNA and RNA synthesis in regenerating rat liver [Yamaguchi and Kanayama, 1996; Yamaguchi and Ueoka, 1997]. Regucalcin had also inhibitory effect on

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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. 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; Yamaguchi and Nakajima, 1999]. This cell may be a tool to study a role of regucalcin in the regulation of cell proliferation. More recently, it had been shown that regucalcin can inhibit protein tyrosine phosphatase and protein kinase activities which are increased in proliferation of the cloned rat hepatoma cells (H4-II-E) cultured with fetal bovine serum (FBS) [Inagaki and Yamaguchi, 2000; Inagaki and Yamaguchi, 2001]. Regucalcin may play an inhibitory role in signaling pathway which is related to protein phosphatases and protein kinases in proliferative cells, suggesting a suppressive role of regucalcin in cell proliferation. This, however, remains to be elucidated.

The present study, therefore, was undertaken to determine the effect of regucalcin on DNA synthesis in the proliferation of cloned rat hepatoma cells (H4-II-E) stimulated by FBS. We found that an increase in DNA synthesis in the nuclei of cells cultured with FBS precedes cell proliferation, and that the endogenous regucalcin plays a suppressive role in the enhancement of DNA synthesis with cell proliferation.

MATERIALS AND METHODS

Chemicals

α-Minimum essential medium (α-MEM) and penicillin-streptomycin solution (5000 units/ml penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), trifluoperazine (TFP), and sodium orthovanadate were purchased from Sigma (St Louis, MO). S(-1)Bay K8644 was obtained from Research Biochemicals International (Natick, MA). Staurosporine, okadaic acid, PD98059, and cyclosporine A were obtained from Wako Pure Chemical Co. (Osaka, Japan). (Methyl- ^{3}H) deoxythymidine 5'-triphosphate ([^{3}H]-dTTP; 2.59 TBq/mmol) was obtained from New England Nuclear (Boston, MA). The reagents were dissolved in distilled water and ethanol solution.

Isolation of Regucalcin

Male Wistar rats, weighing 100–120 g, were obtained commercially from Japan SLC (Hamamatsu, Japan). Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1993a,b; Yamaguchi and Isogai, 1993]. Regucalcin was isolated from rat liver cytosol. The livers were perfused with Tris-HCl buffer (pH7.4), 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 (w/v) in Tris-HCl buffer (pH 7.4); the homogenate was spun at 5500*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 days later antigen (0.25 mg/animal) was intraperitoneally injected with Freund's incompltete adjuvant. Animals were killed by bleeding 3 days after the last injection. Spleen cells were prepared from immunized mouse and fused into myeloma cells. Anti-regucalin 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 units/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 phosphate-buffered saline (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 sec with an ultrasonic device. Scraped cells were also homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were spun at 100*g* in a refrigerated centrifuge for 5 min to obtain the nuclei. The 100*g* supernatant function was spun at 1000*g* for 10 min, and the precipitated fraction (containing nucleus) was pooled. DNA concentration in the 1000*g* precipitated fraction was determined by the method of Ceriotti [1955].

Determination of Cell Numbers

After trypsinization using 0.2% trypsin plus 0.02% ethylenediamine-tetraacetic acid in Ca^{2+}/Mg^{2+}-free PBS, cell numbers were determined by electronic particle counter.

Estimation of DNA Synthesis

DNA synthesis by the nuclear fraction of H4-II-E hepatoma cells was estimated by the procedure of Lynch et al. [1970] with a minor modification. DNA synthesis was measured for 30 min at 37°C in mixtures (0.5 ml) that contained 0.18 M Tris/HCl buffer (pH 8.2), 4 mM $MgCl_2$, 2 mM ATP, dGTP, dCTP, dATP (each 0.08 mM), 0.06 mM [^{3}H] dTTP, dextran (Type 100 C, 2%, 2.5 mM cadaverine, and the suspension of nuclei (0.1 ml containing 65–80 μg DNA). Regucalcin (10^{-8}–10^{-6} M), anti-monoclonal antibody (25–100 ng/ml) or various inhibitors (PD98059, staurosporine, TFP, okadaic acid, vanadate, and cyclosporin A) were added as indicated. Reactions were stopped with 0.5 ml of 1 M NaOH and DNA was precipitated with addition (5 ml) of ice-cold trichloroacetic acid (10% TCA). The DNA was then dissolved (0.5 ml of 1 M NaOH) and precipitated (5 ml of 10% TCA), and the final precipitate, dissolved in 0.5 ml of 1 M NaOH, was heated at 80°C for 15 min. Finally, the DNA was precipitated with 10% TCA and the precipitate was washed with acid, ethanol, and ether. The radioactivity in nuclear DNA was measured in a hyamine-toluene liquid scintillation mixture, and all the data were corrected for the incorporation with control mixture that lacked the three unlabeled deoxynucleotides. DNA synthesis was expressed as disintegrations per minute (dpm) per milligram of nuclear DNA.

Western Blot Analysis

The homogenate from the cloned rat hepatoma cells cultured with 10% FBS in the absence or presence of Bay K 8644 (2.5×10^{-6} M) was centrifuged for 1 h at 105,000 × *g* at 4°C, and the supernatant (cytosol) was used for Western blot analysis [Wessendorf et al., 1993]. Aliquots of cytosol were mixed with 5 × Laemmli sample buffer, boiled for 5 min, and SDS–PAGE was performed by the method of Laemmli [1970] using 12% polyacylamide resolving gel. After SDS–PAGE, the proteins were then transferred onto a polyvinylidene difluoride membrane at 100 mA for 4 h. The membranes were incubated with a polyclonal rabbit anti-regucalcin antibody [Yamaguchi and Isogai, 1993], which was diluted 1:2000 in 10 mM Tris-HCl, pH8, containing 150 mM NaCl, 0.1% (w/v) Tween 20 (washing buffer), and 5% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horseradish peroxidase linked anti-rabbit IgG which was diluted 1:5000 with washing buffer containing 5% (w/v) skin milk, and again they were washed. Detection of the protein bands was performed using a enhanced chemiluminescent kit following the manufacture instruction. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel.

Statistical Analysis

Data were expressed as the mean±SEM. The significance of the difference between the values was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. A *P*-value of < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Enhancement of Nuclear DNA Synthesis Activity in Proliferative Cells

The cloned rat hepatoma cells (H4-II-E) were cultured for 6,12,24,48,72, and 96 h in the presence of FBS (1 or 10%). The alteration in cell number and in vitro DNA synthesis activity in the nuclear fraction of cell homogenate is shown in Figure 1. A significant increase in cell number was seen at 24 h after culture with 10% FBS. Such an increase was not observed by the

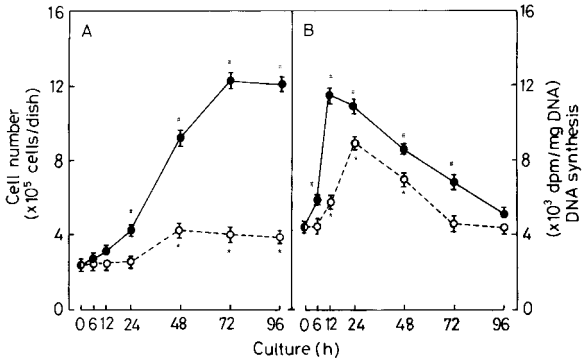

Fig. 1. Changes in cell numbers and nuclear DNA Synthesis activity of the cloned rat hepatoma cells (H4-II-E) cultured with different concentration of FBS. Cells (2.5×10^5) were cultured for 6, 12, 24, 48, 72, and 96 h in the presence of FBS (1.0 or 10%). DNA synthesis activity was measured using the nuclear fraction of cell homogenate. 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.

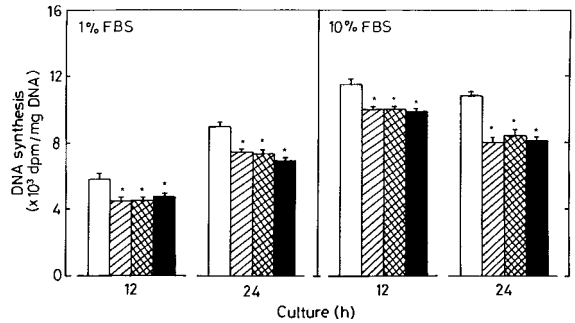

Fig. 2. Effect of protein kinase inhibitors on DNA synthesis activity in the nuclei of the cloned rat hepatoma cells (H4-II-E) cultured with different concentrations of FBS. Cells were cultured for 12 and 24 h in the presence of FBS (1.0 or 10%). DNA synthesis activity was measured in a reaction mixture containing either vehicle, PD98051 (10^{-5}M), staurosporine (10^{-6} M) or trifluoperazine (TFP; 10^{-3} M) using the nuclear fraction of cell homogenate. Each value is the mean±SEM of five experiments. *$P < 0.01$, compared with the control (none) value. White bars, control; hatched bars, PD98051; double hatched bars, staurosporine; black bars, TFP.

culture with 1% FBS. Cell number, however, was significantly raised 48–96 h after culture with 1% FBS. Meanwhile, DNA synthesis activity in the nuclear fraction of cell homogenate was significantly increased 6 h after culture with 10% FBS and elevated markedly 12 h after the culture, and then began to decrease between 24–96 h. The cell culture with 1% FBS caused a significant increase in DNA synthesis activity at 12 h after the culture, and the activity was reached to maximun at 24 h and then began to decrease. An increase in DNA synthesis activity in the cells preceded an elevation of cell number in the culture with 1 or 10% FBS.

The effect of various protein kinase inhibitors on in vitro DNA synthesis activity in the nuclear fraction of hepatoma cells is shown in Figure 2. Cells were cultured for 12 and 24 h in the presence of 1 or 10% FBS. DNA synthesis activity in the nuclei of cells cultured for 12 and 24 h with FBS (1 or 10%) was significantly inhibited in the presence of PD98059 (10^{-5} M), an inhibitor of MAP kinase [Zhang et al., 1999], staurosporine (10^{-6} M), an inhibitor of protein kinase C [Tamaoki et al., 1986], or trifluoperazine (TFP; 10^{-5} M), an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase [Vincenzi, 1982], in the reaction mixture (Fig. 2). Meanwhile, the presence of various protein phosphatase inhibitors (10^{-5} M okadaic acid, 10^{-5} M vanadate, and 10^{-5} M cyclosporine A) in the reaction mixture did not cause a significant changes in nuclear DNA synthesis activity in the cells cultured with FBS (1 or 10%) for 12 or 24 h (data not shown).

Regulatory Role of Regucalcin in the Enhancement of Nuclear DNA Synthesis Activity in Proliferative Cells

The effect of regucalcin on in vitro DNA synthesis activity in the nuclear fraction of the

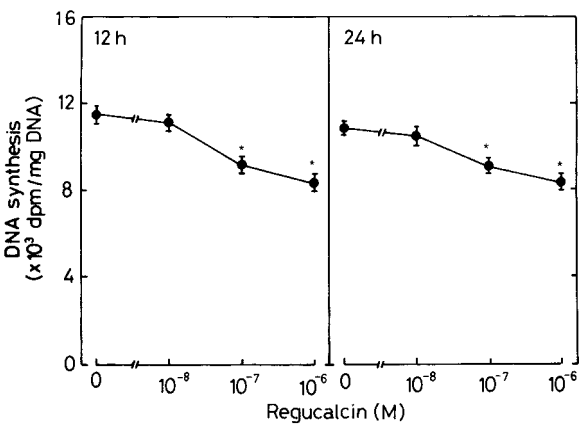

Fig. 3. Effect of regucalcin on DNA synthesis activity in the nuclei of the cloned rat hepatoma cells (H4-II-E) cultured with 10% FBS. Cells were cultured for 12 and 24 h in the presence of 10% FBS. DNA synthesis activity was measured in a reaction mixture containing either vehicle or regucalcin (10^{-8}–10^{-6} M) using the nuclear fraction of cell homogenate. Each value is the mean±SEM of five experiments. *$P < 0.01$, compared with the control (none) value.

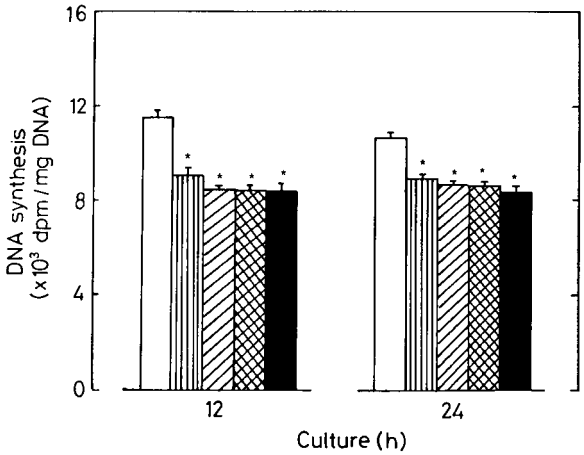

Fig. 4. Effect of protein kinase inhibitors on the regucalcin-decreased DNA synthesis activity in the nuclei of the cloned rat hepatoma cells (H4-II-E) cultured with 10% FBS. DNA synthesis activity was measured in a reaction mixture containing either vehicle, PD98051 (10^{-5} M), staurosporine (10^{-6} M) or TFP (10^{-5} M) without or with regucalcin (10^{-7} M) using the nuclear fraction of cell homogenate. Each value is the mean ± SEM of five experiments. *$P < 0.01$, compared with the control (none) value. White bars, control (none); lined bars, regucalcin; hatched bars, regucalcin plus PD98051; double hatched bars, regucalcin plus staurosporine; black bars, regucalcin plus TFP.

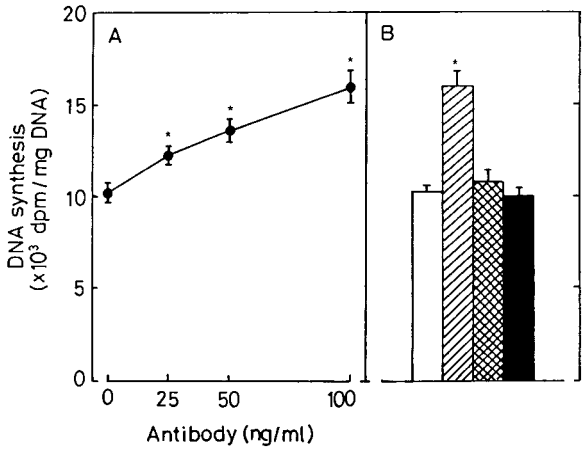

Fig. 5. Effect of anti-regucalcin monoclonal antibody on DNA synthesis activity in the nuclei of the cloned rat hepatoma cells (H4-II-E) cultured with 10% FBS. DNA synthesis activity was measured in a reaction mixture containing (**A**) either vehicle or anti-regucalcin monoclonal antibody (ARMA; 25, 50, and 100 ng/ml), or (**B**) either vehicle, ARMA (100 ng/ml), ARMA (100 ng/ml) plus regucalcin (10^{-6} M) or none immune IgG (100 ng/ml). Each value is the mean ± SEM of five experiments. *$P < 0.01$, compared with the control (none) value. White bars, control (none); hatched bars, ARMA; double hatched bars, ARMA plus regucalcin; black bars, none immune IgG.

cloned rat hepatoma cells is shown in Figure 4. Cells were cultured for 12 and 24 h in the presence of 10% FBS. DNA synthesis activity was significantly decreased by the addition of regucalcin (10^{-7} or 10^{-6} M) in the reaction mixture containing the nuclei of cells cultured with FBS for 12 and 24 h. The reduction was not significantly altered in the presence of varius protein kinase inhibitors (10^{-5} M PD98059, 10^{-6} M staurosporine, or 10^{-5} M TFP) in the reaction mixture with the addition of regucalcin (10^{-7} M) (Fig. 4).

The effect of anti-regucalcin monoclonal antibody on in vitro DNA synthesis activity in the nuclear fraction of the cloned rat hepatoma cells is shown in Figure 5. Cells were cultured for 24 h in the presence of 10% FBS. DNA synthesis activity was significantly elevated in the presence of anti-regucalcin monoclonal antibody (25, 50, and 100 ng/ml) in the reaction mixture containing the nuclei of cells (Fig. 6A). The effect of anti-regucalcin monoclonal antibody (100 ng/ml) in increasing DNA synthesis activity was completely prevented by the addition of regucalcin (10^{-6} M) in the reaction mixture (Fig. 6B). Meanwhile, none immune IgG (100 ng/ml) did not have an appreciable effect on DNA synthesis activity.

The effect of various protein kinase inhibitors on the anti-regucalcin monoclonal antibody-increased DNA synthesis activity in the nuclear fraction of the cloned rat hepatoma cells is shown in Figure 6. Cells were cultured for 24 h in the presence of 10% FBS. The effect of anti-

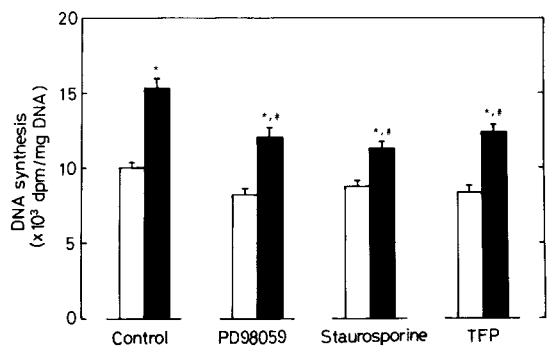

Fig. 6. Effect of protein kinase inhibitors on anti-regucalcin monoclonal antibody-increased DNA synthesis activity in the nuclei of the cloned rat hepatoma cells (H4-II-E) cultured with 10% FBS. Cells were cultured for 24 h in the presence of 10% FBS. DNA synthesis activity was measured in a reaction mixture containing either vehicle, PD98051 (10^{-5} M), staurosporine (10^{-6} M) or TFP (10^{-5} M) in the absence or presence of anti-regucalcin monoclonal antibody (100 ng/ml). Each value is the mean ± SEM of five experiments. *$P < 0.01$, compared with the control value. #$P < 0.05$, compared with the value for anti-regucalcin antibody alone. White bars, none; black bars, anti-regucalcin antibody.

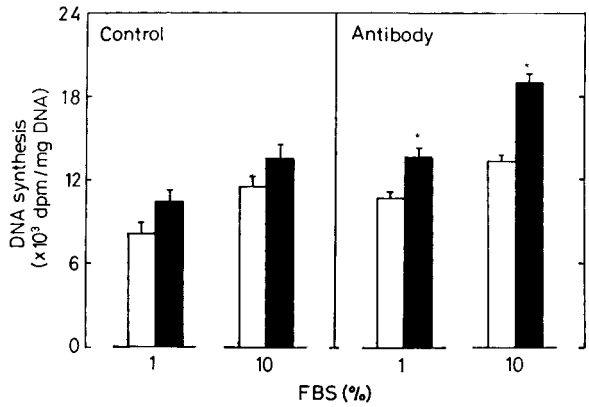

Fig. 7. Effect of anti-regucalcin monoclonal antibody on DNA synthesis activity in the nuclei of the cloned rat hepatoma cells (H4-II-E) cultured with Bay K 8644, an agonist of Ca^{2+} entry in cells. Cells were cultrued for 24 h in the presence of either vehicle or Bay K 8644 (2.5×10^{-6} M) with 1.0 or 10% FBS. DNA syntheis sactivity was measured in a reaction mixture containing either vehicle or anti-regucalcin monoclonal antibody (100 ng/ml). Each value is the mean ± SEM of five experiments. $^*P < 0.01$, compared with the control (none) value. White bars, control; black bars, Bay K 8644.

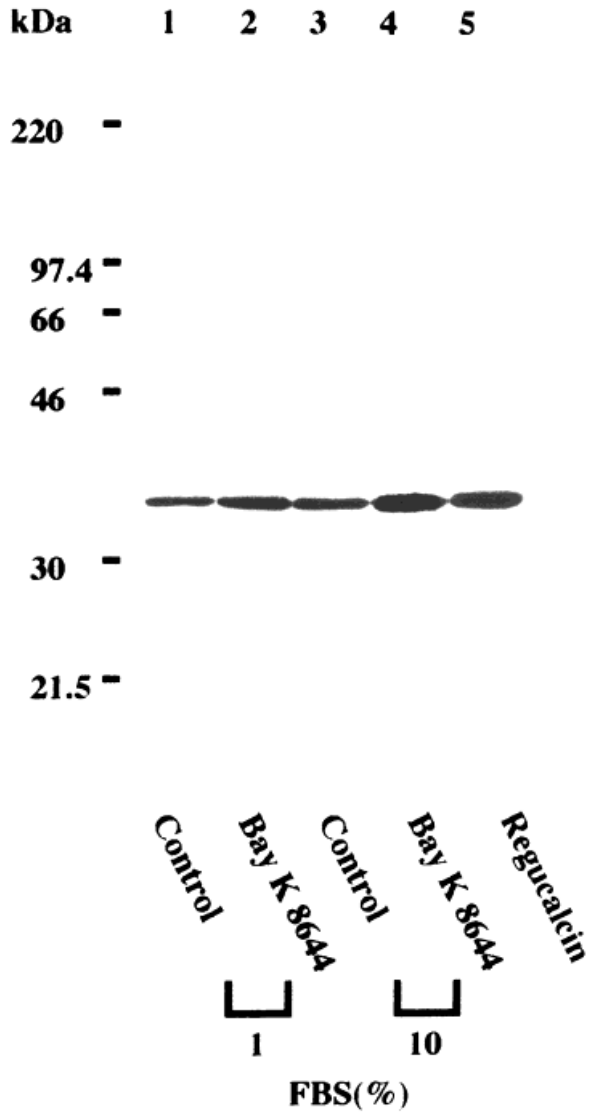

Fig. 8. Changes in regucalcin levels in the cytosol of the cloned rat hepatoma cells (H4-II-E) cultured with Bay K 8644, an agonist of Ca^{2+} entry in cells. Cells were cultured for 24 h in the presence of either vehicle or Bay K8644 (2.5×10^{-6} M) with FBS (1.0 or 10%). Western blot analysis was carried out on the extracts (25 μg of cytosolic proteins) obtained from the hepatoma cells. With 1%FBS, **lane 1**-control, **lane 2**-Bay K8644; with 10% FBS, **lane 3**-control, **lane 4**-Bay K 8644; **lane 5**, regucalcin (0.1μg) as the marker. The figure shows 1 of 3 experiments with separate samples.

regucalcin monoclonal antibody (100 ng/ml) in increasing DNA synthesis activity was significantly inhibited in the presence of PD98059 (10^{-5} M), staurosporine (10^{-6} M) or TFP (10^{-5} M) in the reaction mixture. These inhibitory effects, however, were partial.

Effect of Bay K 8644 on Nuclear DNA Synthesis Activity in Proliferative Cells

The effect of Bay K 8644, an agonist of Ca^{2+} entry into cells, on DNA synthesis activity in the nuclear fraction of the cloned rat hepatoma cells is shown in Figure 7. Cells were cultured for 24 h with FBS (1 or 10%) in the presence of Bay K 8644 (2.5×10^{-6} M). DNA synthesis activity was not significantly altered by culture with Bay K 8644. The presence of anti-regucalcin monoclonal antibody (100 ng/ml) in the reaction mixture containing the nuclei of cells cultured with Bay K 8644 caused a significant increase in DNA synthesis activity.

The alteration in regucalcin levels in the cloned rat hepatoma cells cultured with Bay K 8644 is shown in Figure 8. Cells were cultured for 24 h with FBS (1 or 10%) in the presence of either vehicle or Bay K 8644 (2.5×10^{-6} M). Western blot analysis for regucalcin showed that culture with Bay K 8644 caused a significant ($P < 0.01$) increase in regucalcin levels in the cells cultured with 1 or 10% FBS; the densitometric data with Bay K 8644 treatment showed 151±5.1 or 131±3.8 (% of control; mean ± SEM of three experiments) as compared with that of control value obtained from cells cultured with 1 or 10% FBS, respectively.

DISCUSSION

This study demonstrates that an increase in nuclear DNA synthesis activity precedes an

elevation of the number of the cloned rat hepatoma cells (H4-II-E) cultured with FBS (1 or 10%), and that the nuclear DNA synthesis is suppressed by endogenous regucalcin, suggesting that regucalcin regulates DNA synthesis in the nuclei of proliferative cells. Regucalcin has been shown to transport in the nucleus of rat liver [Tsurusaki et al., 2000], and it can inhibit nuclear DNA synthesis of normal rat liver and regenerating rat liver [Yamaguchi and Kanayama, 1996]. Regucalcin in the nucleus may play a suppressive role in the proliferation of liver cells.

DNA synthesis activity in the nuclei of cloned rat hepatoma cells (H4-II-E) was remarkable at 12 and 24 h after culture with FBS. This increase was significantly inhibited in the presence of PD98059, an inhibitor of MAP kinase, staurosporine, an inhibitor of protein kinase C, and TFP, an antagonist of Ca^{2+}/calmodulin-dependent protein kinase, in the reaction mixture. An increase in nuclear DNA synthesis activity by serum stimulation may be partly mediated through action of various protein kinases in the nuclei of hepatoma cells. However, serum stimulation-induced increase in nuclear DNA synthesis activity did not seem to be related to action of various protein phosphatases.

The presence of regucalcin in the reaction mixture caused a significant decrease in DNA synthesis activity in the nuclei of cloned rat hepatoma cells cultured with FBS. This effect was not significantly altered in the presence of various protein kinase inhibitors. Regucalcin has been demonstrated to inhibit the activity of various protein kinases in the nuclei of liver cells [Inagaki and Yamaguchi, 2001; Katsumata and Yamaguchi, 1998]. The effect of regucalcin in decreasing nuclear DNA synthesis activity may be partly mediated through the pathway of various protein kinases in the cloned rat hepatoma cells.

DNA synthesis activity was significantly increased in the presence of anti-regucalcin monoclonal antibody in the reaction mixture containing the nuclei of cloned hepatoma cells cultured for 24 h with 10% FBS, supporting that endogenous regucalcin in the nucleus has an inhibitory effect on DNA synthesis activity. This elevation was significantly inhibited by the addition of various protein kinase inhibitors in the reaction mixture. This inhibition by inhibitors, however, was partial. From these results, it is assumed that the effect of endogenous regucalcin in inhibiting DNA synthesis activity in the nuclei is involved in protein kinase inhibition and other mechanisms. Presumably, regucalcin has a direct-partial inhibiting effect on DNA synthesis activity in the nuclei of cloned rat hepatoma cells.

The transcriptional activity for regucalcin gene has been shown to be enhanced in the cloned rat hepatoma cells cultured with Bay K 8644, an agonist of Ca^{2+} entry in cells, in the presence of 10% FBS [Yamaguchi and Nakajima, 1999; Misawa and Yamaguchi, 2000]. Culture with Bay K 8644 caused a significant increase in regucalcin levels in the cloned hepatoma cells in the presence of FBS (1 and 10%). In this case, nuclear DNA synthesis activity was not significantly changed by culture with Bay K 8644. The presence of anti-regucalcin monoclonal antibody in the reaction mixture containing the nuclei of cells cultured with Bay K 8644 resulted in a significant elevation of nuclear DNA synthesis activity. Nuclear DNA synthesis activity is suppressed by endogenous regucalcin which was raised in the nuclei of hepatoma cells cultured with Bay K 8644. Regucalcin may have a physiologic role in the regulationof nuclear DNA synthesis activity in proliferative cells.

Regucalcin has been shown to inhibit DNA synthesis, protein kinase and protein phosphatase activities in the nuclei of regenerating rat liver with proliferative cells [Yamaguchi and Kanayama, 1996; Katsumata and Yamaguchi, 1998; Omura and Yamaguchi, 1999], and it could inhibit DNA synthesis in the nuclei of cloned rat hepatoma cells with proliferation after serum stimulation. Regucalcin may play a suppressive role in the proliferation of liver cells, because of regulating over expression of cell proliferatin.

In conclusion, it has been demonstrated that endogenous regucalcin has an inhibitory effect on DNA synthesis in the nuclei of cloned rat hepatoma cells (H4-II-E) cultured with serum stimulation.

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