MODULATION OF INSULIN-STIMULATED DNA SYNTHESIS BY CHOLERA TOXIN IN BOVINE MAMMARY FIBROBLASTS

I. S. Yuh[1], C. K. Park, J. Y. Han[2] and L. G. Sheffield[3]

Department of Dairy Science, College of Animal Agriculture
Kangwon National University, Chuncheon 200-701, Korea

Summary

Bovine fibroblasts were cultured in Dulbecco's Modified Eagle's Medium and then treated with control, insulin (I, 1 μg/ml), cholera toxin (CT, 0.1-100 ng/ml) or CT (0.1-100 ng/ml) + I (1 μg/ml). Cholera toxin, an activator of adenylate cyclase, significantly decreased insulin induced DNA synthesis ($p < 0.05$). The modulation of DNA synthesis apparently involves events occurring in early stage of cell growth, at least between the first 4 and 8 hour of CT treatment. Insulin induced collagen as well as noncollagen synthesis in cell layer, however, these syntheses were reduced by addition of cholera toxin ($p < 0.05$) but were not completely reduced. It is not clear whether the reduction of insulin-induced cell layer collagen or noncollagen proteins by CT is involved in the inhibitory effect on insulin-induced DNA synthesis. However, we could rule out the hypothesis that insulin-induced DNA synthesis is reduced by CT-induced cellular differentiation.

(**Key Words** : Bovine Fibroblast, Cholera Toxin, Insulin, DNA Synthesis)

Introduction

Cyclic 3', 5'-adenosine monophosphate (cAMP) has been implicated in variety of cellular processes, including growth and differentiation. Cholera toxin (CT) stimulates adenylate cyclase activity and increases intracellular cyclic AMP (Holmgreen, 1981). CT increases the growth of mammary epithelia in vitro (Yang et al., 1980; Stampfer, 1982) and promotes mammary gland development when systemically injected into intact mice (Sheffield et al., 1985). Interestingly, depending on culture conditions and type of target cell, cAMP has been found to be either growth inhibitory or growth stimulatory (Pastan et al., 1975). The addition of cAMP to the culture medium inhibits the growth of transformed cells (Perkins and Macintyre, 1971) and growing non-transformed 3T3 cells (Bombik and Burger, 1973). Heldin et al. (1989) reported that forskolin, a stimulator of adenylate cyclase activity, inhibited the growth response to platelet-derived growth factor (PDGF) of human foreskin fibroblast in culture. Previously, CT has been shown to augment bovine mammary epithelial growth (Sheffield, 1989) but effects on non-epithelial mammary tissue have not been examined.

The objective of this study is to understand the modulation of insulin-induced DNA synthesis by cAMP-dependent pathway in bovine fibroblasts.

Materials and Methods

Cell isolation and culture :

Fresh bovine mammary tissue was minced into small pieces, placed in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., MO, USA) containing collagenase (0.01%) and incubated overnight at 5% CO_2 and 95% air at 37℃. After pipetting to dissociate tissue and filtration through sterile gauze, pellets were washed 3 times in HBSS (Sigma Chemical Co., MO, USA) and resuspended in DMEM supplemented with 10% horse serum (Sigma Chemical Co., Mo, USA). 3.5 mg/ml of penicillin and 50 mg/ml of streptomycin. For experimental purpose, 3×10^4 bovine mammary fibroblasts (BMFs) were subcultured in 60 mm Falcon tissue

culture dishes (Becton Dickinson Labware, NJ, USA) with 5 ml DMEM containing 10% horse serum for 24 h. BMFs were arrested in G0/G1 by incubation in serum free DMEM for 24 h.

Experiment 1 : Dose response

G0/G1 arrested BMFs in 60 mm tissue culture dishes were incubated with insulin (I μg/ml, I; Sigma Chemical Co., MO, USA), CT (.1-100 ng/ml; Sigma Chemical Co., MO, USA), I + CT or serum free DMEM (control) for 20 h and pulsed for 1 h with [³H]-thymidine (78.5-85.6 Ci/mmol; NEN Research Products, DE, USA). The radioactivity (0.5 μCi/ml) incorporated into trichloroacetic acid (TCA)-precipitable material was measured as follow: cells were washed 3 times using Tris buffer (50 mM, pH 7.5) containing 150 mM NaCl, fixed using ice cold 10% TCA for 15 min, washed 3 times using 100% ethanol, dried under room temperature, and solubilized in .5M sodium hydroxide- .01% triton X-100. Incorporated radioactivity was determined by liquid scintillation.
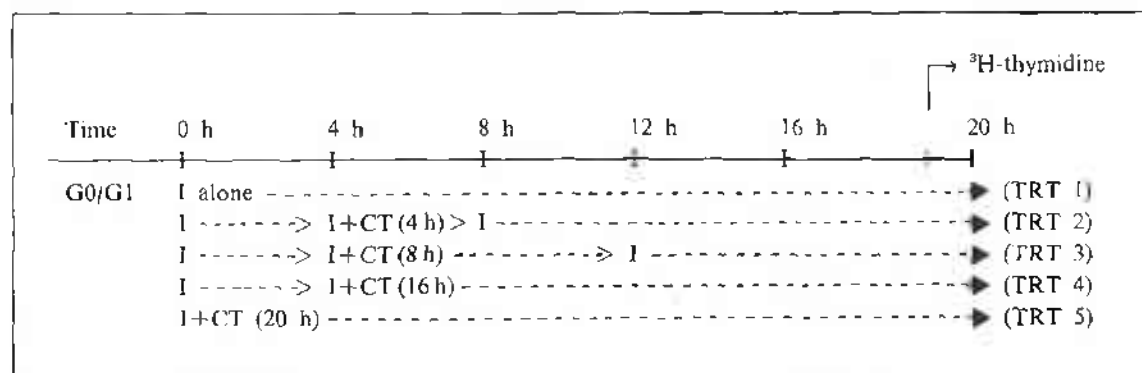
Experiment 2 :

1) Time of CT addition : Dishes were divided into 6 treatments : 20, 16, 12, 8 or 4 h incubation group with CT (1 ng/ml), and zero-CT for 20 h as control, all containing insulin (1 μg/ml, 20 h incubation). CT was added at time 0, 4, 8, 12 or 16 h, and DNA synthesis was measured at 20 h, as described in Experiment 1.
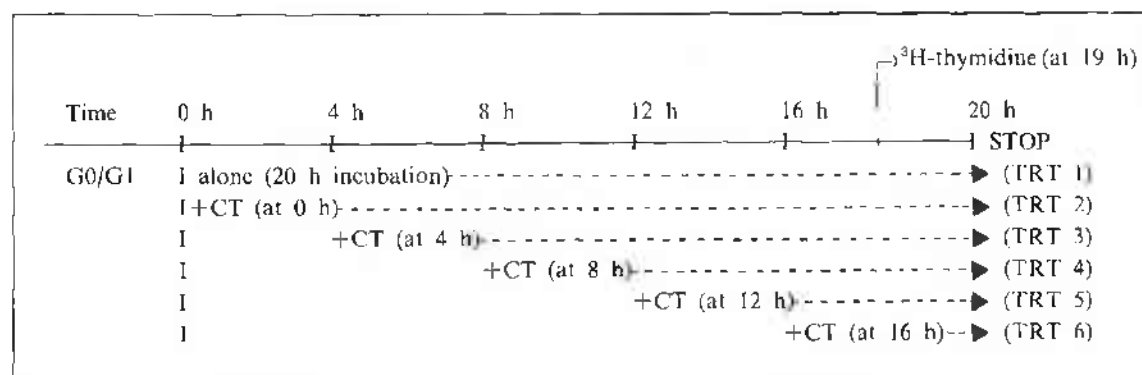
2) Short term incubation of CT at the early stage of cell growth: At time 0, BMFs in all groups were treated with I (1 μg/ml), and only one group (20 h CT incubation group) was supplemented with CT (1 ng/ml). At 4 h, CT was added to 4, 8 and 16 h CT incubation groups. At 8 h, DMEM containing I or CT was changed, refreshed with DMEM and I, and then CT was added again to 8, 16 and 20 h incubation groups and removed from the 4 h CT incubation group. At 12 h, DMEM containing I or CT was changed, refreshed with DMEM and I, and then CT was added to 16 or 20 h CT incubation groups and removed from the 8 h group. [³H]-thymidine incorporation into BMFs was measured as described in Experiment 1.

Experiment 3 :

1) Collagen and noncollagen synthesis : Routine cell culture procedure, medium and supplements were same as described previously but all BMFs arrested in G0/G1 in serum free DMEM were refreshed with DMEM right before treatment. BMFs in 60 mm tissue dishes were incubated with I (1 μg/ml), CT (1 ng/ml), I + CT or serum free DMEM (control) for 24 h. [^{3}H]-proline (25-55 Ci/mmol) were purchased from NEN Research Products (Wilmington Co., DE, USA). Cells were pulsed for whole incubation (24 h) with [^{3}H]-proline (0.5 μCi/ml) after treatment. Cell layer and media were separated from 60 mm tissue culture dishes. Cells which were attached in 60 mm tissue culture dishes were scraped by rubber policemen and rinsed 3 times with 150 mM NaCl and transfered into disposable glass tubes.

2) Protein separation : Proteins from each tube of medium or cell layer were precipitated with 3 volumes of ice cold 10% TCA solution for 1 h and then centrifuged (1,500 g, 30 min and 4 ℃). The resulting protein pellets were washed three times with 3 volumes of cold 5% TCA solution to remove any unincorporated amino acids. The washed pellets were then extracted with three volumes of 5% TCA in a sealed tube for 60 min at 90℃ to solubilize collagen (Newman and Langer, 1975). After centrifugation (1,500 g, 30 min and 4℃). the TCA hydrolysate was extracted 3 times with water saturated isobutanol and its radioactivity was determined by liquid scintillation.

3) DNA assay : DNA concentration per each dish was determined by fluorometric assay as described by Labarca and Paigen (1980).

4) Statistical analysis : Data were analyzed by analysis of variance as a completely randomized block design with more than one observation per experimental unit by Stat View (Brain Power, Inc., 1986). Planned comparisons were used to estimate the effects of various treatments. All comparisons were 2-sided and differences were considered significant at a 5% level (Snedecor and Cochran, 1987).

Results

Experiment 1 : Dose Response to CT

The addition of CT to cell cultures resulted in an inhibition of DNA synthesis, whether stimulated by insulin (1 μg/ml) or not (figure 1). In the absence of insulin, a response significantly different from that in zero-CT and zero insulin control (no addition control) was achieved with .1, 1, 10 or 100 ng/ml CT (p < 0.05). These doses of CT resulted in DNA synthesis values that were 23.2, 13.6, 19.2 or 42.4% of no addition control. In the presence of insulin, .1, 1, 10 or 100 ng/ml CT resulted in DNA synthesis values that were 44.7, 48.9, 55.0 or 76.7% of insulin alone (all less than insulin alone, p < 0.05). No significant differences among .1, 1 or 10 ng/ml CT could be seen with or without insulin (p > 0.05).
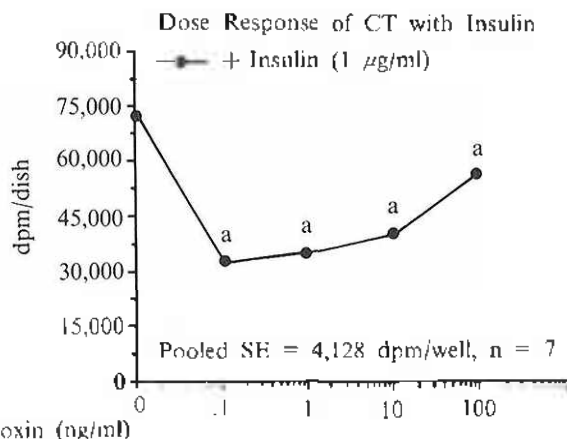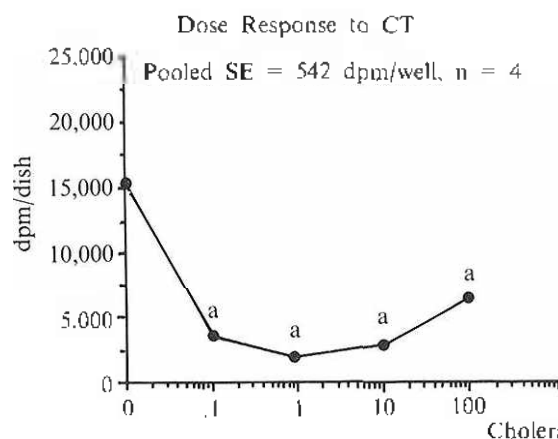

Figure 1. Effect of cholera toxin (CT) on DNA synthesis by bovine fibroblasts in the absence or presence of insulin (1 μg / ml). Statistical comparisons: a = significant differences relative to control in the absence of insulin or presence of insulin (p < 0.05). n = the number of wells per each treatment.

Experiment 2 : Time of CT addition and short term incubation of CT at the early stage of cell growth

The inhibitory effects of CT (1 ng/ml) on [³H]-thymidine incorporation of BMFs depended on time of CT addition (figure 2). Addition of CT at time 0, 4 or 8 h (CT present for the final 20, 16 or 12 h of culture) decreased DNA synthesis (values were 33.6, 41.9 or 68.3% of insulin alone, $p < 0.05$). Addition of CT at a latter time (12 or 16 h after initiation of cultures; corresponding to the final 8 or 4 h of culture) did not affect DNA synthesis at 20 h after culture initiation (values of 91.7 and 91.7% of insulin alone, $p > 0.05$). To further examine the time course of the inhibitory effect of CT on BMFs DNA synthesis, CT was added at 4 h after insulin addition, then removed at 8 and 12 h. The presence of CT between 4 and 8 h (4 h incubation), between 4 and 12 h (8 h incubation), between 4 and 20 h (16 h incubation) and between 0 and 20 h (20 h incubation) after insulin addition resulted in DNA synthesis values of 41.9, 50.2, 43.2 and 28.2% of that in the absence of CT ($p < 0.05$) and no effect of continuous 8 or 16 h CT incubation compared to that of 4 h incubation, could be seen (figure 3, $p > 0.05$). CT is not necessarily needed between the first 4 and 8 hour of incubation period in order to depress DNA synthesis. The inhibitory effect of CT should be involved in earlier time than this period.

Experiment 3 : Collagen and noncollagen synthesis

From the previous result, we could not rule out the possibility of cellular differentiation by CT resulting in the inhibitory effect of insulin-induced DNA synthesis. To examine the possibility of cellular differention by CT, the effect of CT on insulin-induced noncollagen and collagen protein synthesis in media and cell layer was determined by assaying [³H]-proline incorporation into TCA extracts and pellets. None of the treatments significantly altered the noncollagen and collagen protein released into the media (figure 4, $p > 0.05$). However, insulin alone induced 1.9 fold increase of noncollagen and 2.8 fold increase of collagen in the cell layer compared to each control ($p < 0.05$, figure 5). Addition of CT decreased insulin-induced collagen synthesis of cell layer ($p < 0.05$) but did not completely decrease.
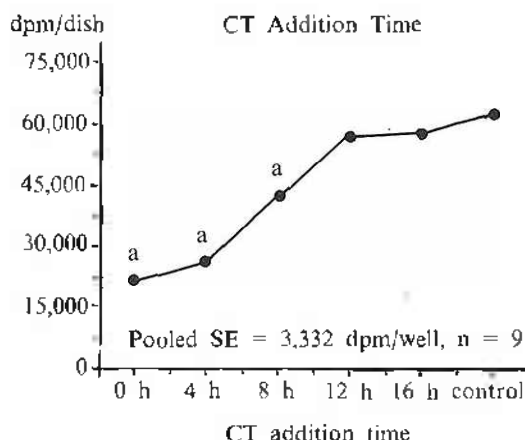

Figure 2. Effect of time of CT addition (1 ng / ml) on insulin-induced ³H-thymidine incorporation by bovine fibroblasts. a = significant effect of CT to decrease insulin-induced DNA synthesis at a given time of CT addition ($p < 0.05$). n = the number of wells per each treatment.
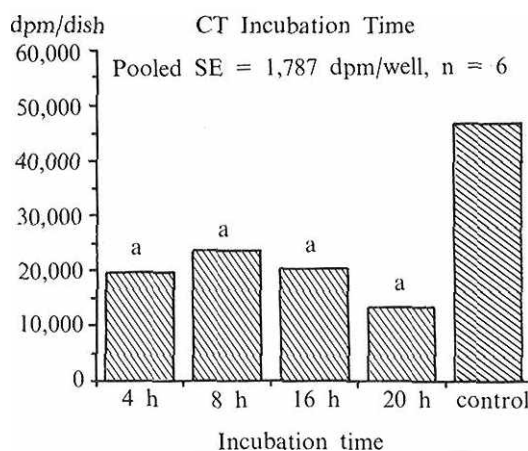

Figure 3. Effect of duration of CT (1 ng / ml) treatment on insulin-induced (1 μg / ml) DNA synthesis by bovine mammary fibroblasts. a — significant effect of CT to decrease insulin-induced DNA synthesis for given duration of CT treatment ($p < 0.05$). treatment: *Insulin alone between 1 20 h, 20 h incubation (control); CT plus insulin between 0-20 h, 20 h incubation; CT plus insulin between 4-8 h, 4 h incubation; CT plus insulin between 4-12 h, 8 h incubation; CT plus insulin between 4-20 h, 16 h incubation.* n = the number of wells per each treatment.

Media Collagen

Media Noncollagen

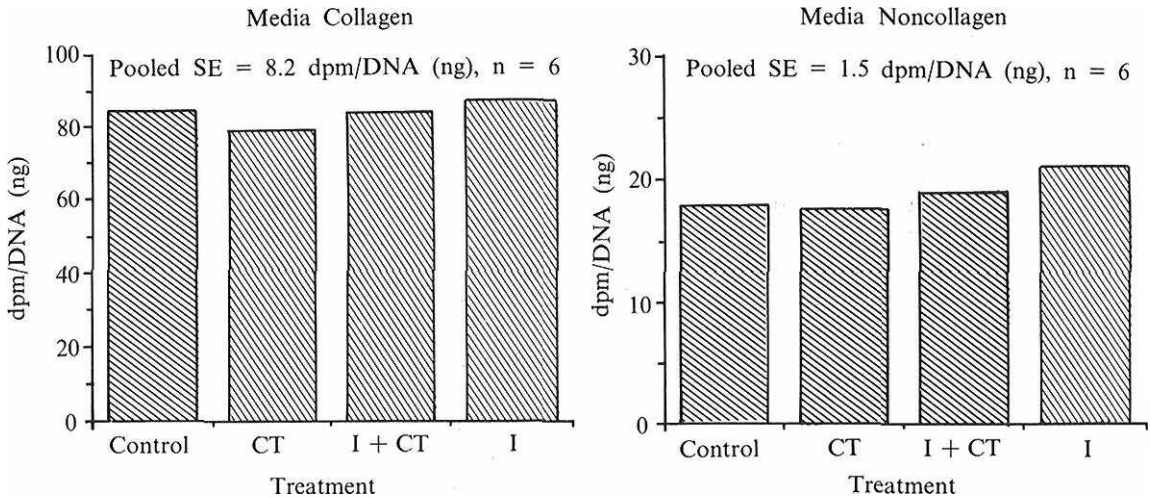Pooled SE = 8.2 dpm/DNA (ng), n = 6

Pooled SE = 1.5 dpm/DNA (ng), n = 6

Figure 4. Effect of insulin (I), CT or I + CT on ³H-proline incorporation into collagen or noncollagen in media. Induction of media collagen or non collagen by treatments was separately analysed by ANOVA. Effects are not significant relative to control in induction of media collgen or noncollagen (p > 0.05). n = the number of wells per each treatment.
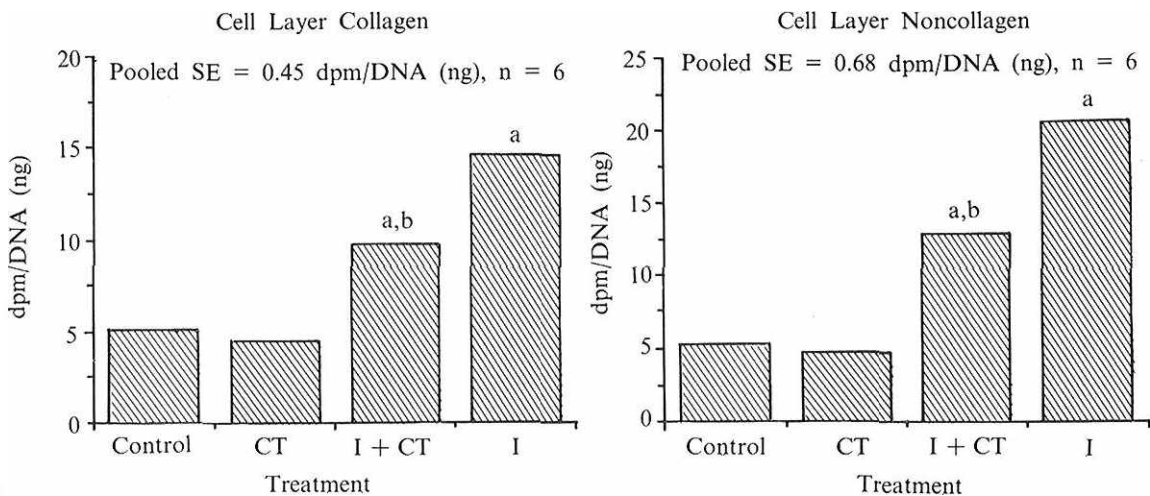
Cell Layer Collagen

Cell Layer Noncollagen

Pooled SE = 0.45 dpm/DNA (ng), n = 6

Pooled SE = 0.68 dpm/DNA (ng), n = 6

Figure 5. Effect of I, CT or I + CT on ³H-proline incorporation into collagen or noncollagen in cell layer. Induction of cell layer collagen or noncollagen by treatment was separately analysed by ANOVA. Statistical comparisons: a = significantly different from control (p < 0.05); b = significantly different from insulin (p < 0.05). n = the number of wells per each treatment.

Discussion

Cholera toxin (CT) increases intracellular cAMP concentrations through its effects on G stimulatory protein (Gs) and by stimulation of adenylate cyclase (Cassel and Pfeuffer, 1978). The possible involvement of cAMP in the regulation of normal cell growth has been subject of several investigations, and both mitotic and inhibitory effects, depending primarily on cell type and culture conditions, have been shown (Gottesman and Fleischmann, 1986). cAMP analogs and other agents that increase the intracellular level of cAMP introduce the proliferation of mammary epithelial cells (Sheffield et al., 1985; Imagawa et al., 1988; Yuh and Sheffield, 1991) and Swiss

3T3 cells (Rozengurt et al., 1983). However, a growth inhibitory effect has been recorded in normal fibroblasts (Heldin et al., 1989), vascular smooth muscle cells (Nilsson and Olsson, 1984) and B lymphocytes (Blomhoff et al., 1987).

The present results show that CT inhibits the growth-stimulatory effect of insulin in cultures of bovine fibroblasts. The simultaneous addition of CT (0.1-100 ng/ml) and insulin (1 μg/ml) led to inhibition of the subsequent initiation of [³H]-thymidine incorporation. In addition, the inhibitory effect of CT on [³H]-thymidine incorporation of bovine fibroblasts depended on time of CT addition (decreases with addition of CT at time 0, 4 or 8 h, $p < 0.05$) and apparently involved events occurring in early stage of cell growth, at least between the first 4 and 8 hour of CT treatment.

Collagen is a major component of the extracellular matrix regulating growth and differentiation of variety of cells. Initially, we could not rule out the possibility that CT differentiates bovine fibroblasts with increasing cellular collagen protein and inhibits insulin-induced DNA synthesis. Our results observed that insulin stimulates the accumulation of collagen and noncollagen protein in the cell layer. Insulin stimulates the expression of type I and type III collagen mRNA and induces synthesis of media and cell layer collagen in cultures of quiescent human lung fibroblasts (Goldstein et al., 1989). In diabetic animals not treated with insulin, the collagen content of the skin is decreased, suggesting that insulin may also contribute the normal regulation of the extracellular matrix (Andressen et al., 1981). Those previous results were similar to the increase of insulin induced collagen production in bovine fibroblast. In otherwise, CT reduced insulin-induced collagen and noncollagen protein in the cell layer, even CT did not affect collagen synthesis by itself. These results rule out the hypothesis that CT differentiates bovine mammary fibroblast with increasing cellular collagen protein. It has been reported that cAMP increase the degradation of newly synthesized collagen in human lung fibroblasts (Baum et al., 1980) and inhibit collagen synthesis in osteoblast cell line (Rosen and Luben, 1983). Those results suggest that CT in our experiment should inhibit insulin-induced collagen synthesis or reduce collagen stability which might be necessary for normal

regulation of cellular growth in bovine fibroblast. In the cell layer, the inhibitory effect of CT on insulin-induced collagen and noncollagen protein synthesis was highly compatible to the inhibitory effect of DNA synthesis by CT on cell layer. At this time, it is not clear whether the reduction of insulin-induced cell layer collagen or noncollagen proteins by CT is involved in the inhibitory effect on insulin-induced DNA synthesis. However, we could rule out the hypothesis that insulin-induced DNA synthesis is reduced by CT-induced cellular differentiation. We need to further examine whether CT directly interfere insulin stimulated growth pathway or not. Insulin like growth factor (IGF-I) possesses approximately 50% homology with proinsulin, and has mitogenic effect in mammary tissue (Oka et al., 1991). In our experiment, insulin maximized growth response of bovine fibroblast growth at pharmacological concentration (1 μg/ml). Thus, it remains to be seen whether the growth stimulatory effect by insulin was mediated through IGF-I receptors or not, and whether the growth inhibitory effect of CT in insulin-induced DNA synthesis was involved in IGF-I receptor mediated-pathways.

In summary, cholera toxin, an activator of adenylate cyclase, inhibits insulin-induced DNA synthesis in bovine fibroblasts. The inhibition of DNA synthesis occurs early stage of insulin-induced bovine fibroblast growth. CT reduced insulin-induced collagen and noncollagen protein in the cell layer, even CT did not affect collagen synthesis by itself. These studies suggest that cAMP dependent pathway should be at least an inhibitory modulator of insulin or by itself in bovine fibroblasts proliferation.

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