The effects of menaquinone-4 and its structurally related compound on steroidogenesis and insulin secretion

博士論文

The effects of menaquinone-4 and its structurally related compound on steroidogenesis and insulin secretion.

(メナキノン-4、および構造類縁体がステロイド産生、およびインスリン分泌に与える影響)

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Introduction

Discovery of vitamin K

Vitamin K (VK) was discovered by Henrik Dam in 1929 while studying the role of cholesterol by feeding chicks a low cholesterol diet. The chicks then developed hemorrhages and started bleeding, and this condition cannot be improved by feeding purified cholesterol. To figure out the cause, Dam further found another compound in diet which prevented the bleeding. Dam called the compound vitamin K from "Koagulations-vitamin" in German and Scandinavian languages (1).

In the last several decades, VK were isolated and characterized. VK is a family of fat-soluble compounds with a common chemical structure, 2-methyl-1,4-naphthoquinone (also known as menadione). Naturally existing VK includes vitamin K1 (VK1, phylloquinone) and vitamin K2 (VK2, menaquinone). VK1 is highly found in plant origin, especially in green leafy vegetables which was first isolated from alfalfa (2). However, VK2 were isolated from putrefied fishmeal (3) that VK2 are primarily of bacterial origin. VK2 are differ in structure from VK1 in their unsaturated polyprenyl side chain (Fig. 1A and B), the major menaquinones contain 4-10 isoprenoid units, involve menaqinone-4 (MK-4, Fig. 1C) to MK-10. MK-4 is not commonly synthesized by bacteria, it is alkylated from animal feeds which contain menadione (Fig. 1D) or is converted from dietary VK1 and other menaquinones (4, 5). MK-7 is found in a great amount in fermented soybean (natto) and certain cheeses (6). MK-4 is composed of 2-methyl-1,4-naphthoquinone and a geranylgeranyl side chain that is considered the effector component rather than the naphthoquinone ring of MK-4.

Geranylgeraniol (GGOH) is a C20 isoprenoid compound (Fig. 1E) found in fruits,

vegetables, and grains (7) that plays an important role in several biological processes, and has been also considered as a functional side chain component of MK-4.

Functions of vitamin K

VK is well-known as a nutrient required for blood coagulation and bone metabolism. As a matter of fact, it is used as a therapeutic agent for osteoporosis and preventing fractures in Japan (8, 9). VK is a cofactor of γ-glutamyl carboxylase (GGCX) which is needed to the formation of γ-carboxyglutamyl (Gla) residues from glutamate residues (Glu) in VK-dependent proteins (10), these proteins are found in various types of cell and tissue. It is worth mentioning here, recent studies revealed that VK regulates protein kinase A (PKA) activation in different cell lines. Tsang *et al*. reported that VK enhances nerve growth factor-mediated neurite outgrowth via the activation of PKA in PC12D pheochromocytoma cells (11). VK2 also inhibits the growth and invasion of hepatocellular carcinoma cells through the activation of PKA (12). Furthermore, VK2 modulates the target genes by induction of the activation of PKA in osteoblastic cells (13).

So far, there is a long-range interests in the reaction of VK in different tissues in our laboratory. Pervious study, Minegishi (14) found that the expressions of steroid-related genes enhanced in VK1-supplemented rats than VK-deficient rats by using DNA microarray analysis. By using qRT-PCR, the enzyme in the first step of steroidogenesis, Cyp11a, also showed the positive correlation with MK-4 concentrations in the rat testis. Furthermore, testosterone level and Cyp11a protein level reduced in VK-deficient rats, it revealed that VK deficiency is linked to suppress the steroidogenesis.

Moreover, Ito (15, 16) described MK-4 enhances testosterone production *in vivo* and *in vitro* experiments. In MK-4 supplemented rats, plasma and testis testosterone levels were significantly increased compared to those of control rats. MK-4 but not VK1 also enhanced testosterone production in testis-derived cells. Western blot analysis revealed that MK-4 enhanced the expression of Cyp11a and phosphorylation levels of PKA and the cAMP response element-binding protein.

Functions of GGOH— the structurally related compound of MK-4

On the other hand, not only MK-4 exhibits extensively functions that GGOH also showed the benefit in biological processes. To base on previous studies, they showed that GGOH may also have anti-tumorigenic effects against leukemia (17), prostate cancer (18), colon cancer (19) cells, and anti-inflammatory activity in rats (20). However, the detailed mechanisms underlying these effects remain unclear. Recent studies of our laboratory also found that both MK-4 and GGOH have anti-inflammatory activity in lipopolysaccharide (LPS)-induced inflammation model. Ohsaki found that VK suppressed the inflammatory cytokines though inhibited the activation of nuclear factor κB (NF-κB) in macrophage-like cells (20). Giriwono also found that GGOH improved LPS-induced inflammation via the inhibition of NF-κB activation in rats (21). Another *in vitro* study, Yoshida (22) found that testosterone and progesterone production enhanced in a time- and dose-dependent manner by the presence of GGOH. Moreover, GGOH regulated adenylate cyclase (AC) and PKA activity in testis-derived I-10 cells. Furthermore, Hirahara found that MK-4 and GGOH induced insulin secretion in a dose- and time-dependent manner after stimulated with glucose in cultured mouse pancreatic islets (23).

Based on these considerable experimental evidences, we highlight the importance of MK-and GGOH not only for their widely known cofactor function but also for other novel functions in biological processes. According to our previous studies, we hypothesized that MK-4 and GGOH may act an important role in cAMP/PKA signaling pathway.

Steroidogenesis and testosterone production in testis

In men, testosterone is produced by Leydig cells in the testicles. As shown in Fig. 2, steroidogenic acute regulatory protein (StAR) transports cholesterol to the inner mitochondrial membrane in these cells to initiate steroidogenesis. P450scc (also known as CYP11A), a cholesterol side chain cleavage enzyme, catalyzes a cascade of reactions that converts cholesterol to the steroid hormone precursor pregnenolone, which is converted to testosterone. Steroid synthesis is also regulated by multiple signaling events, including AC activation and elevation of intracellular cAMP levels, followed by activation of PKA and other downstream steroidogenic proteins (24).

Testosterone plays an important role in fetal development, sperm production, and the development of male secondary sex characteristics in men. A population-based study showed that free serum testosterone levels decrease with age in Japanese men aged 40–79 years (25). Low testosterone levels cause infertility and sexual dysfunction in men, with a subset of men developing late-onset hypogonadism. Low testosterone can also predict the development of type 2 diabetes and cardiovascular disease and has been linked to increased risk of mortality in men (26-30).

Glucose stimulated-insulin secretion

Another important issue now is that type 2 diabetes is a common and serious global health problem and is increasing rapidly worldwide. Type 2 diabetes frequently results from progressive failure of pancreatic β-cell function in the presence of insulin resistance. Current issues suggest appropriate glycemic control is one of the most important goals in patient management (31, 32). The response of insulin secretion from pancreatic β-cells after an increase in the blood glucose concentration plays a critical role in glycemic control.

As shown in Fig. 3, it is a complicated metabolic mechanism by which glucose stimulation of insulin release. Under the normal condition, when the extracellular glucose concentration increases, pancreatic β-cell metabolism then accelerates that lead to an increase of ATP concentration. As a result of these metabolic changes, the increased ATP leads to closing of the ATP-sensitive K^+ channels in the plasma membrane. The resulting decrease in K^+ leads to depolarization of the cell with subsequent opening of Ca^{2+} channel which activates exocytosis of insulin granules and leads to insulin secretion in β-cell (33). Thus, cAMP levels are hardly affected by glucose stimulation. And incretins can amplify insulin signaling after meal by elevating intracellular cAMP level.

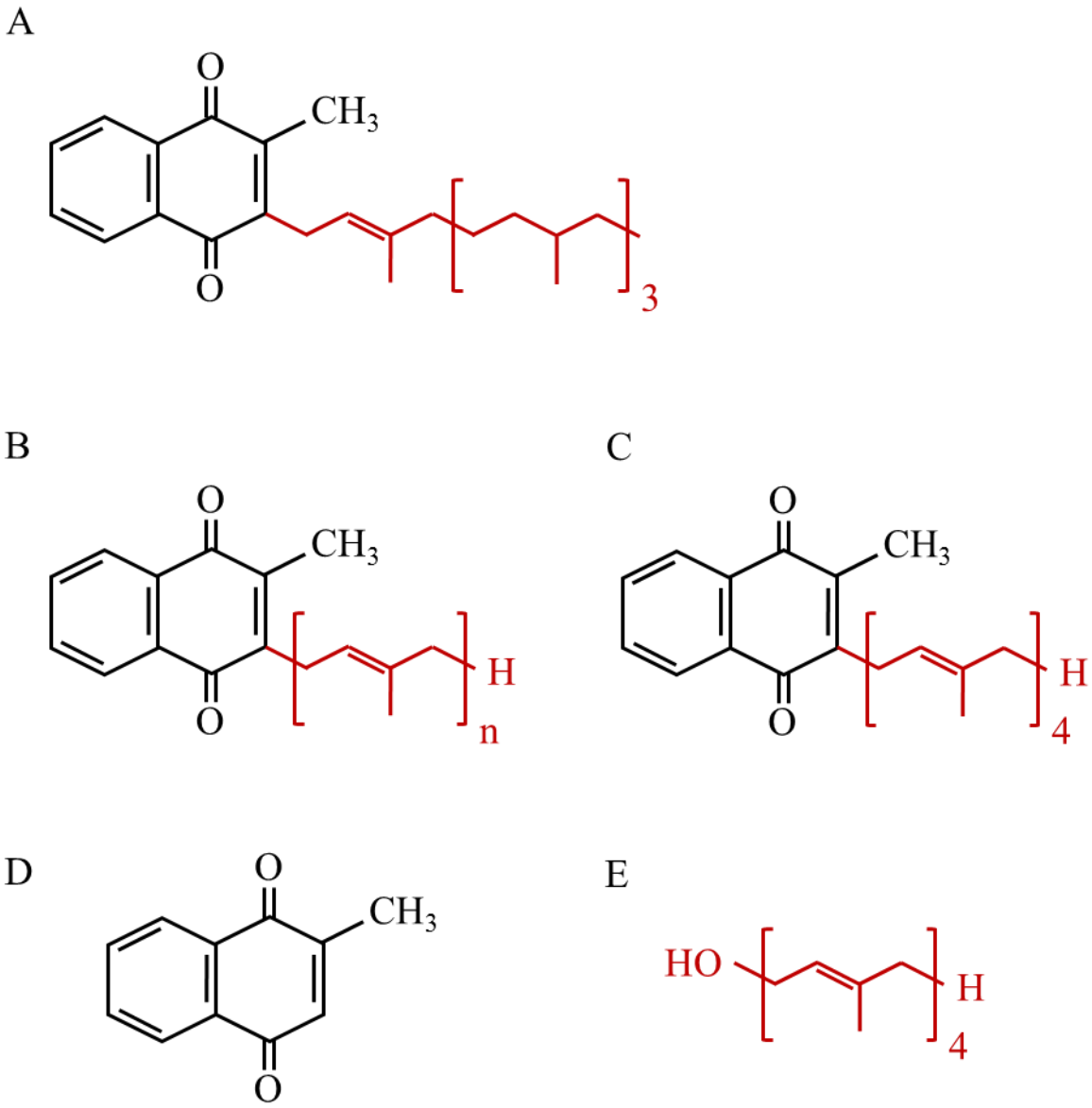


Fig. 1. Chemical structures of vitamin K and geranylgeraniol. (A) vitamin K1 (VK1, phylloquinone), (B) vitamin K2 (VK2, menaquinone) (C) menaqinone-4 (MK-4) (D) menadione (E) geranylgeraniol (GGOH).

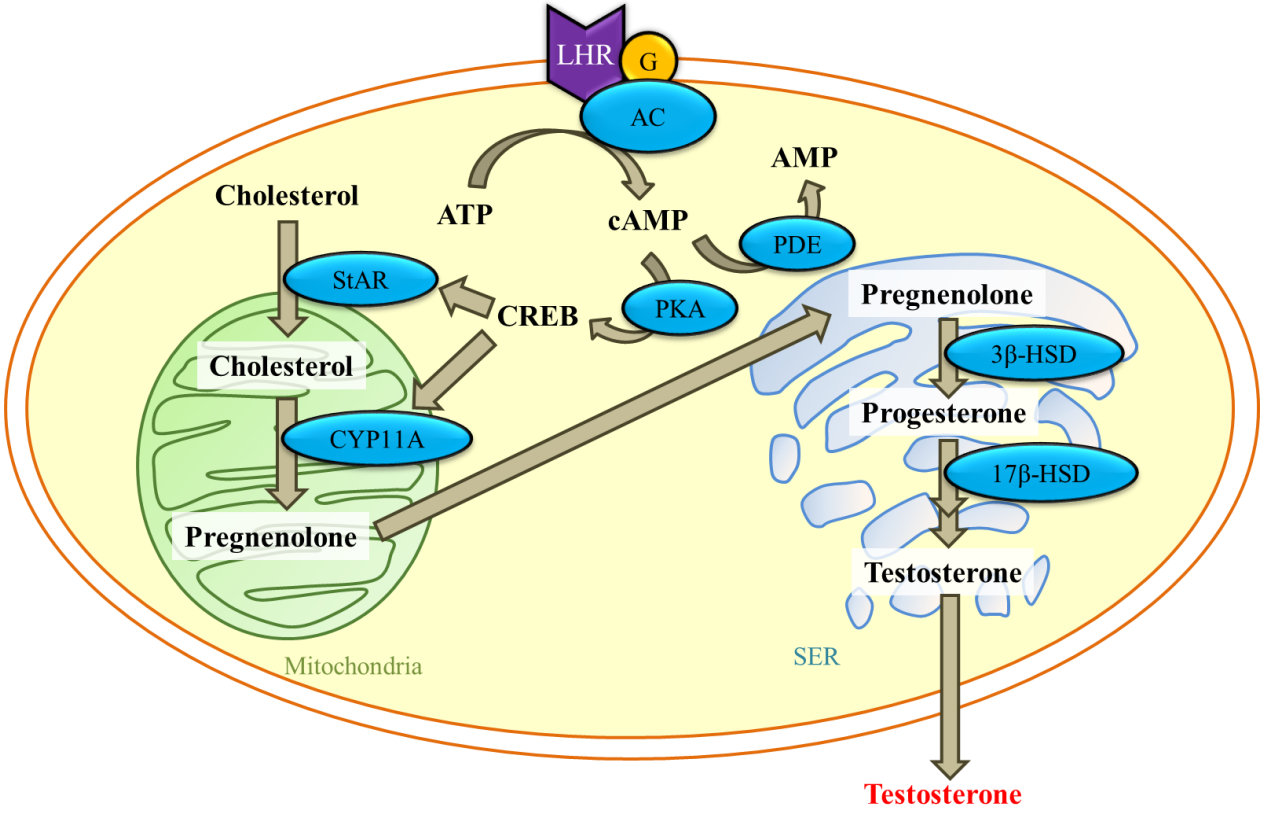


Fig. 2. Steroidogenesis pathway in Leydig cells.

LHR, Luteinizing hormone receptor; AC, adenylate cyclase; PDE, phosphodiesterase; PKA, protein kinase A; CREB, cAMP response element-binding protein; StAR, steroidogenic acute regulatory protein; CYP11A, cholesterol side-chain cleavage enzyme; HSD, hydroxysteroid dehydrogenase.

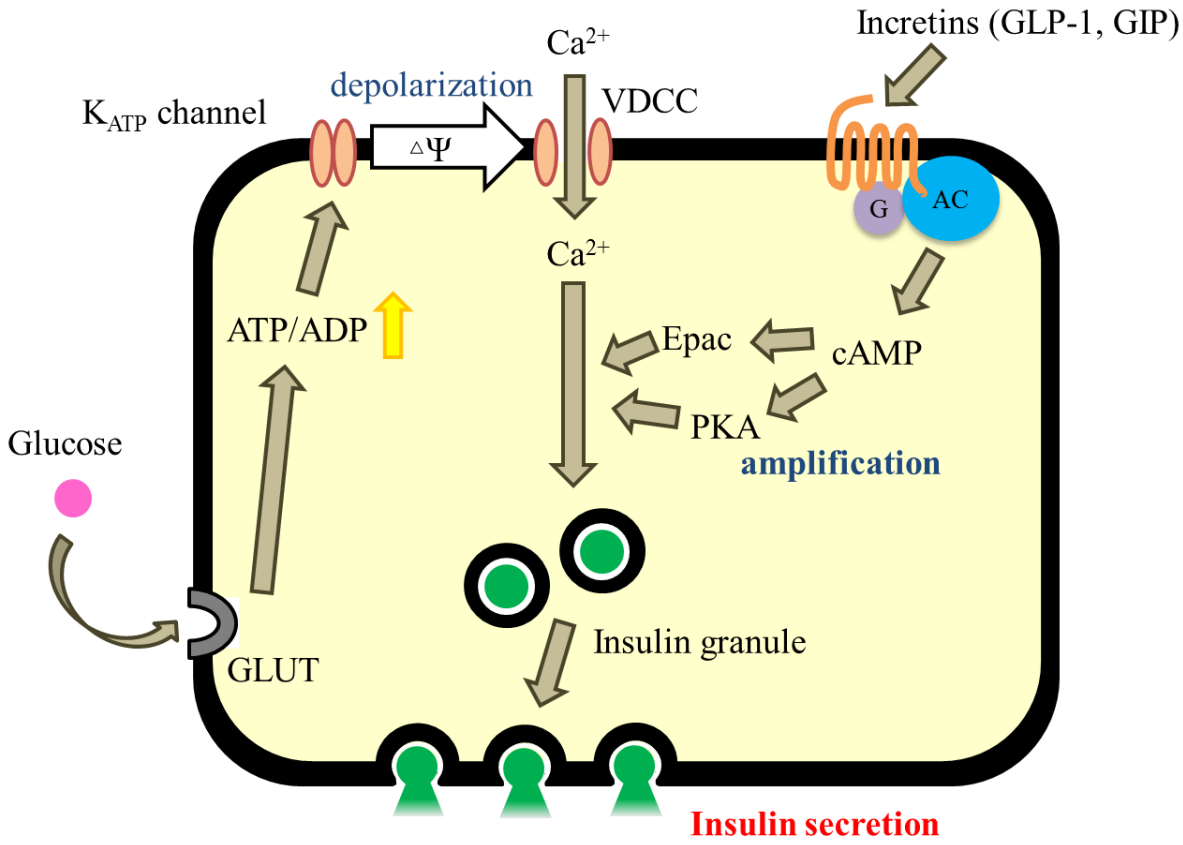


Fig. 3. Glucose-stimulated insulin secretion and incretin-induced amplification pathway in pancreatic β-cell. AC, adenylate cyclase; G, G protein; GLUT, glucose transporter; VDCC, voltage dependent calcium channel.

Chapter 1 MK-4 and GGOH stimulate steroidogenesis by modulating PKA and AC activities but not phosphodiesterase (PDE) in a cAMP-dependent manner in I-10 cells

We previously showed that MK-4 and GGOH can enhance testosterone production in rats and in mouse testis-derived I-10 tumor cells. Here, we further clarified the mechanism of enhanced testosterone and progesterone production by MK-4 and GGOH in the present study by using I-10 cells.

Section 1 Effects of MK-4 and GGOH on testosterone and progesterone productions

First we analyzed the testosterone and progesterone concentrations in the culture medium to reconfirm the steroidogenesis response of MK-4 and GGOH in I-10 cells.

Experimental procedures

Cell line and culture conditions

Cell line: I-10 mouse testis-derived tumor cells were acquired from the Health Science Research Resources Bank (Osaka, Japan).

Culture medium: I-10 cells were maintained in Ham's F-10 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 50 U/ml penicillin, and 50 μg/ml streptomycin.

Culture conditions: I-10 cells were cultured in a 5% CO_2 humidified incubator at

37°C.

Cell passage: Cells were cultured in a 10 cm dish, removed the culture medium by using aspirator, then washed by PBS for twice. 0.25% Trypsin-EDTA was added and incubated for 5 min, then added fresh medium, carefully resuspended cells. Dilute cells with fresh medium in new dish, then place back to incubator. For experiments, cells were used when they reached 80–90% confluence.

Treatments and reagents

MK-4 was obtained from Nisshin Pharma Inc (Tokyo, Japan) and dissolved in ethanol to obtain a stock solution (50 mM). GGOH was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in ethanol to obtain a stock solution (100 mM). MK-4 and GGOH were dissolved in ethanol at 50 mM and 100 mM, respectively, and then were stored protected from light at -20°C. γ-Tocotrienol (γ-T3) was obtained from Prof. Miyazawa, Tohoku University. γ-T3 was dissolved in ethanol to obtain a stock solution (16.2 mM).

Testosterone and progesterone levels in culture medium

I-10 cells were seeded in 12-well plates at a density of 6.0×10^4 cells/well and incubated overnight. The culture medium was then replaced with fresh medium containing MK-4 and GGOH (3, 10, or 30 μM). After incubation for 24 h, the culture medium was collected and centrifuged at 1,000 × *g* for 5 min. Testosterone and progesterone concentrations in the supernatant were determined with specific enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI, USA). The collected mediums were used directly for testosterone, and diluted 10 fold for progesterone

measurement. Measured absorbance using an ELISA reader at 415 nm within 60-90 min. Testosterone and progesterone concentrations were normalized by protein levels. Protein concentrations were measured by using the Lowry method.

Lowry method for protein quantitation

I-10 cells were harvested by scraping, and total cellular protein was prepared in lysis buffer consisting of 50 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.1% SDS and 5 mM EDTA. To 80 μl standard or sample, 400 μl alkaline solution (NaOH and Na_2CO_3 mixture) and $CuSO_4 \cdot 5(H_2O)$ mixed solution (50:1) were added, and incubated at 37°C for 10 min. 40 μl Folin reagent were then added and mixed by a vortex mixer, and incubated at 37°C for 30 min. Read the absorbance 750 nm by using an ELISA reader.

Cell proliferation assay

I-10 cells were seeded into 96-well plate at a density of 1.0×10^4 cells/well and incubated overnight. The medium was replaced the following day with 0, 1, 3, 10, 30, or 100 μM γ-T3. Following incubation for 24 h, the number of viable cells in each sample was determined using the Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan). 10 μl/well Premix WST-1 were added to cells cultured in 100 μl/well (1:10 final dilution), then incubated the cells for 0.5 to 4 h in a 5% CO_2 humidified incubator at 37°C. Measured absorbance using an ELISA reader at 450 nm with a reference wavelength at 630 nm.

Statistical analysis

Data are expressed as mean ± SD and were analyzed with the one-way analysis of

variance followed by Tukey's honestly significant difference test using SAS v.9.3 software (SAS Institute, Cary, NC, USA). A P value < 0.05 was considered significant.

Results and discussion

To determine whether MK-4 and its side-chain structurally related compounds—GGOH enhance testosterone production, the testis-derived tumor cells— I-10 cells were incubated with the treatments of MK-4 and GGOH (0–30μM) for 24 h. The results showed that both MK-4 and GGOH enhanced testosterone level in the culture medium in a dose-dependent manner (Fig. 4A and 4B). Similarly, the production of progesterone—a testosterone precursor—was also increased in a dose-dependent manner following treatments with MK-4 and GGOH (Fig. 4C and 4D). The results showed that MK-4 and GGOH enhance steroidogenesis in I-10 cells.

Moreover, the ability of structurally related isoprenoids to stimulate steroidogenesis was also examined (22). The pervious experiments found that testosterone and progesterone levels in I-10 cells culture medium were markedly increased in the presence of isoprenoids— phytol and geranylgeranyl diphosphate; meanwhile, farnesol enhanced progesterone but not testosterone level. In Fig. 5, we used γ-tocotrienol (γ-T3) in current study, it is the member of vitamin E family and has a farnesyl isoprenoid side chain that we considered that γ-T3 may enhance steroidogenesis. For a start, to determine the dosage of γ-T3, we analyzed the effect of different concentrations of γ-T3 on cell toxicity in I-10 cells, and it showed that when the concentrations of γ-T3 under 10 μM is harmless for I-10 cells (Fig. 5A); therefore, we next used 0.3-3 μM γ-T3 to investigate the effects of γ-T3 on testosterone and progesterone levels (Fig. 5B and 5C), the results revealed that γ-T3 enhanced

progesterone level in a dose-dependent manner. And it is consistent with the effects of farnesol in the previous study (22), progesterone but not testosterone levels were increased by γ-T3 treatments in a dose-dependent manner.

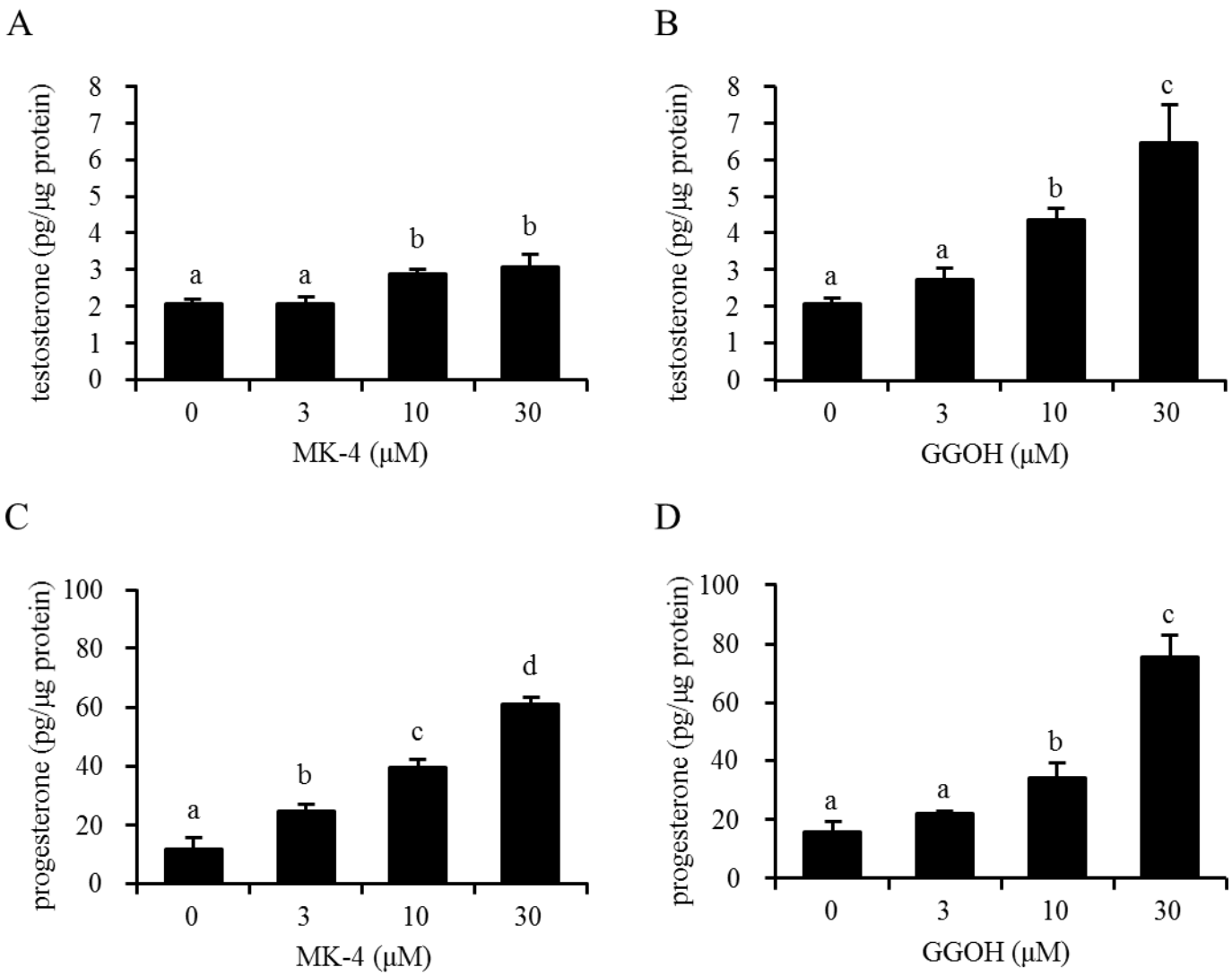


Fig. 4. MK-4 and GGOH stimulates steroidogenesis. MK-4 (A, C) and GGOH (B, D) enhance testosterone and progesterone production in I-10 cells, respectively. Cells were treated with indicated concentrations of MK-4 or GGOH for 24 h. Testosterone and progesterone levels in the culture medium were measured by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

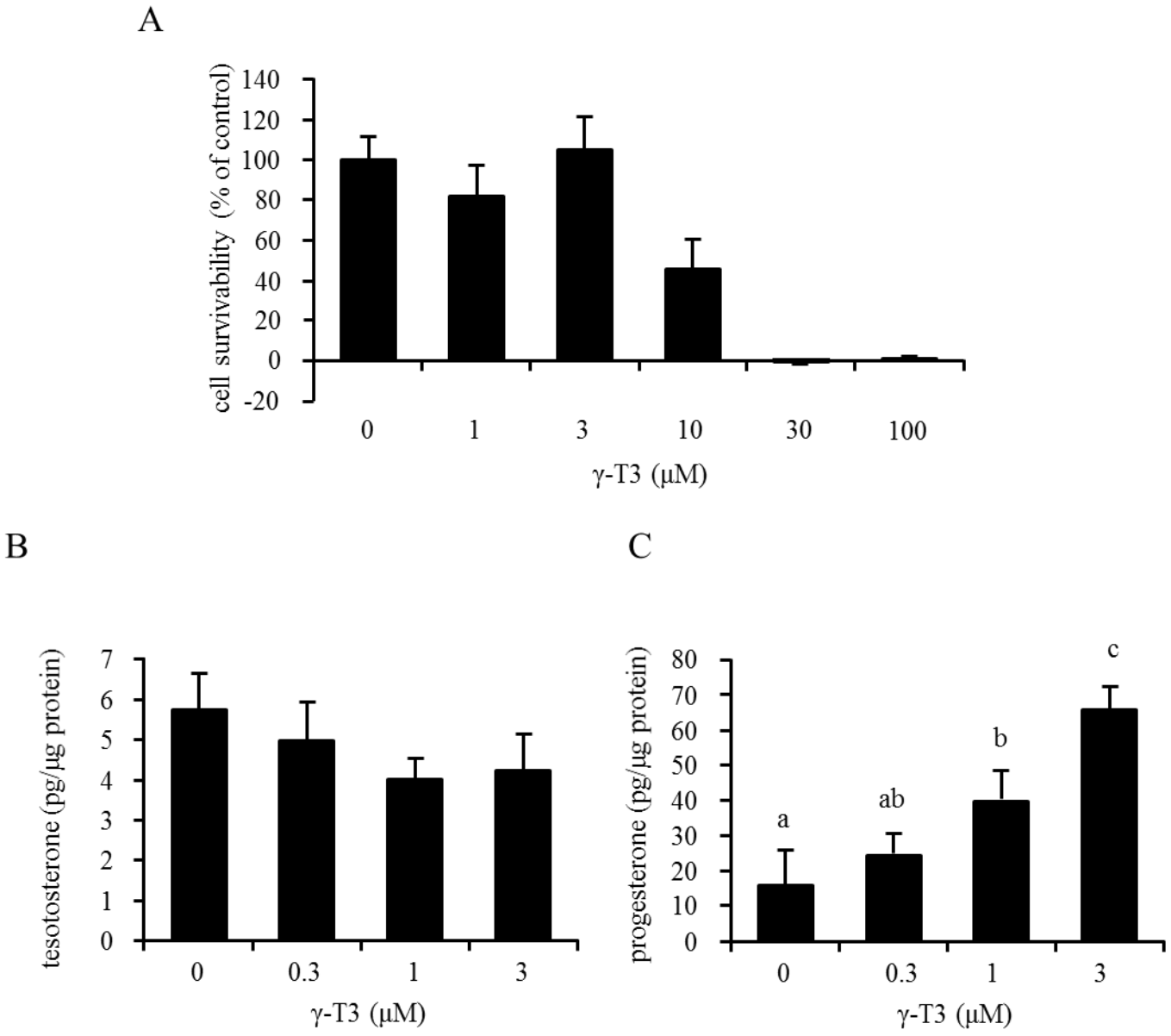


Fig. 5. T3 stimulates steroidogenesis. (A) Effect of T3 on cytotoxicity in I-10 cells. Data are presented as mean ± SD (n=6). γ -T3 stimulated steroidogenesis. Effects of γ-T3 on testosterone (A) and progesterone (B) productions in I-10 cells. Cells were treated with indicated concentrations of MK-4 and GGOH for 24 h. Testosterone and progesterone levels in the culture medium were measured by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

Section 2 Effects of MK-4 and GGOH on cAMP/PKA signaling pathway

Section 1 in this chapter already showed that MK-4 and GGOH stimulate steroidogenesis in I-10 cells. To clarify whether the effects of MK-4 and GGOH on testosterone and progesterone production via the same mechanism in I-10 cells or not, we further investigated the role of MK-4 and GGOH in regulating cAMP/PKA pathway of steroidogenesis in I-10 cells.

Experimental procedures

Cell line and culture conditions

As shown in chapter 1, section 1.

Treatments and reagents

Preparation of MK-4 and GGOH are as shown in chapter 1 section 1. H89 (Sigma-Aldrich), MDL12,330A (Calbiochem, San Diego, CA, USA), and 3-isobutyl-1-methylxanthine (IBMX; Cayman Chemical)—inhibitors of PKA, AC, and phosphodiesterase (PDE), respectively—were dissolved in dimethyl sulfoxide (Sigma-Aldrich) to obtain a stock solution (10 mM).

CRE-Reporter gene assay

FuGENE HD transfection reagent (Promega, Madison, WI, USA) was used to transfect cells with reporter plasmids. Cells were seeded in a 6-well plate and incubated overnight, and the medium was replaced with Opti-MEM (Gibco/Invitrogen, Carlsbad, CA, USA) containing the pGL4.29 and pmiwZ plasmids and transfection reagent. The

pGL4.29 plasmid harboring the firefly luciferase gene under the control of the cAMP response element (CRE) was purchased from Promega; the pmiwZ plasmid containing the β-galactosidase reporter gene was used as an internal control. To incubate for 24 h, fresh medium was exchanged, followed by incubation for another 24 h; cells were then incubated with treatments containing MK-4 and GGOH for 3 h, collected, and lysed with Reporter Lysis Buffer (Promega). Luciferase and β-galactosidase activities were measured with the Luciferase Assay System (Promega) and Galacto-Star β-galactosidase reporter gene system (Invitrogen) by using an AB-2250 luminometer (ATTO, Tokyo, Japan).

Testosterone and progesterone levels in culture medium

As shown in chapter 1, section 1.

Lowry method for protein quantitation

As shown in chapter 1, section 1.

Measurement of cAMP levels

I-10 cells were seeded in 60-mm dishes and incubated overnight. The culture medium was refreshed with MK-4 or GGOH-containing medium, followed by incubation for 1.5 h. Cells were lysed with 0.1 M HCl and centrifuged at 1,000 × *g* for 10 min. The supernatant was acetylated by adding 4 M KOH and acetic acid anhydride before measurement. cAMP concentrations in cell lysates were determined with a cAMP EIA kit (Cayman Chemical).

Statistical analysis.

As shown in chapter 1 section 1.

Results and discussion

As the evidence mentioned above, we considered that MK-4 and GGOH increase steroidogenesis via regulating cAMP/PKA pathway in I-10 cells. To test this hypothesis, we first assessed the effects of MK-4 and GGOH on the activation of PKA. By using the CRE-driven reporter gene assay, we found that 30 μM MK-4 induced luciferase activity and 30 μM GGOH showed a tendency to induce luciferase activity relative to the control in I-10 cells (Fig. 6). On the other hand, the increase in progesterone level induced by MK-4 (Fig. 7A) and GGOH (Fig. 7B) was completely abolished by treatment with the PKA inhibitor H89. Moreover, as shown in Fig. 8, we also examined the luciferase activity after MK-4 and GGOH treatment in different cell line — LS180 (human colon adenocarcinoma cell line) and HepG2 (human liver hepatocellular cells) by using CRE-driven reporter gene assay. The luciferase activity enhanced by the AC activator — forskolin (FSK) in these cell lines but did not regulated by MK-4 or GGOH, these results showed that MK-4 and GGOH modulated cAMP/PKA pathway specific for I-10 cells. Furthermore, intracellular cAMP level was increased in a dose-dependent manner by treatment with MK-4 and GGOH, not γ-T3 for 1.5 h (Fig. 9 and 10). These results indicate that MK-4 and GGOH enhanced testosterone and progesterone production via regulation of PKA activity in a cAMP-dependent pathway.

To clarify the mechanism underlying the effects of MK-4 and GGOH on the cAMP/PKA pathway and steroidogenesis in I-10 cells, we inhibited the activities of two enzymes that regulate cAMP level—i.e., AC and phosphodiesterase (PDE) using

MDL12,330A and IBMX, respectively. Although the effects of 10 and 30 μM MK-4 on progesterone production were blocked in the presence of MDL12,330A, it did not show the significant difference relative to the control (Fig. 11A); however the GGOH-induced increases in progesterone level were suppressed in the presence of MDL12,330A (Fig. 11B). On the other hand, in Fig. 12A and 12B, we first used the low dose (5 μM) of IBMX to clarify the effects of MK-4 and GGOH on PDE activity, the results revealed that both MK-4 and GGOH did not suppress PDE in I-10 cells; even so, to reconfirm the results, in Fig. 13A, IBMX was affirmed as a non-toxic inhibitor under high concentration (50 μM) in I-10 cells. We then used 50μM IBMX to compare the effects of MK-4 and GGOH in the deficiency of PDE activity (Fig. 13B and 13C), and it did not exhibit any significant differences between absence and presence of IBMX, which suggesting that MK-4 and GGOH regulate cAMP concentration by inducing AC activity and not by suppressing PDE.

Moreover, as shown in Fig. 14A, I-10 cells lack hCG/LH receptor that even in a high concentration (1000 μg/mL) of human chorionic gonadotropin (hCG) did not enhance the progesterone production in I-10 cells (Fig. 14B), it is consistent with previous reports which showed that I-10 cells lack hCG/LH receptor (34, 35), thus it revealed that MK-4 and GGOH stimulate progesterone productions not through hCG/LH receptor.

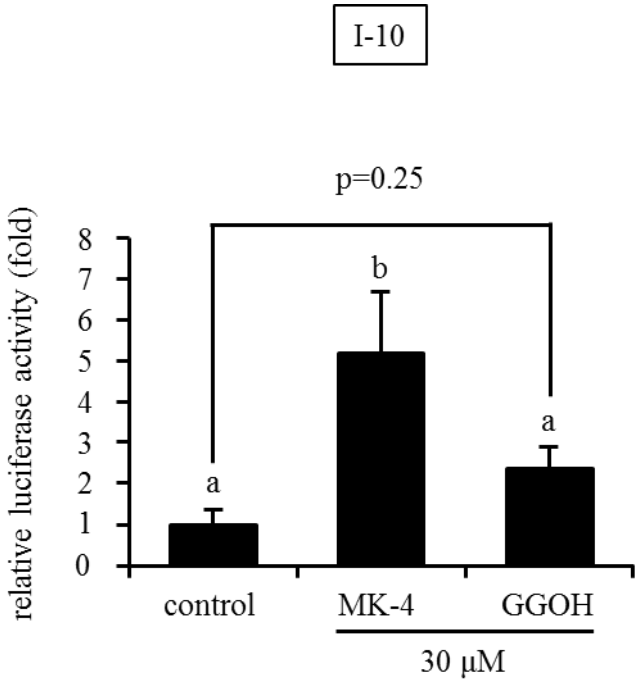


Fig. 6. Effects of MK-4 and GGOH on the luciferase activity in I-10 cells. Cells were transfected with a CRE-inducible reporter gene and then treated with MK-4 or GGOH for 3 h. Reporter activity in cell lysates was measured with the luciferase assay. Data are presented as mean ± SD (n=3). Different letters indicate significant differences ($P < 0.05$).

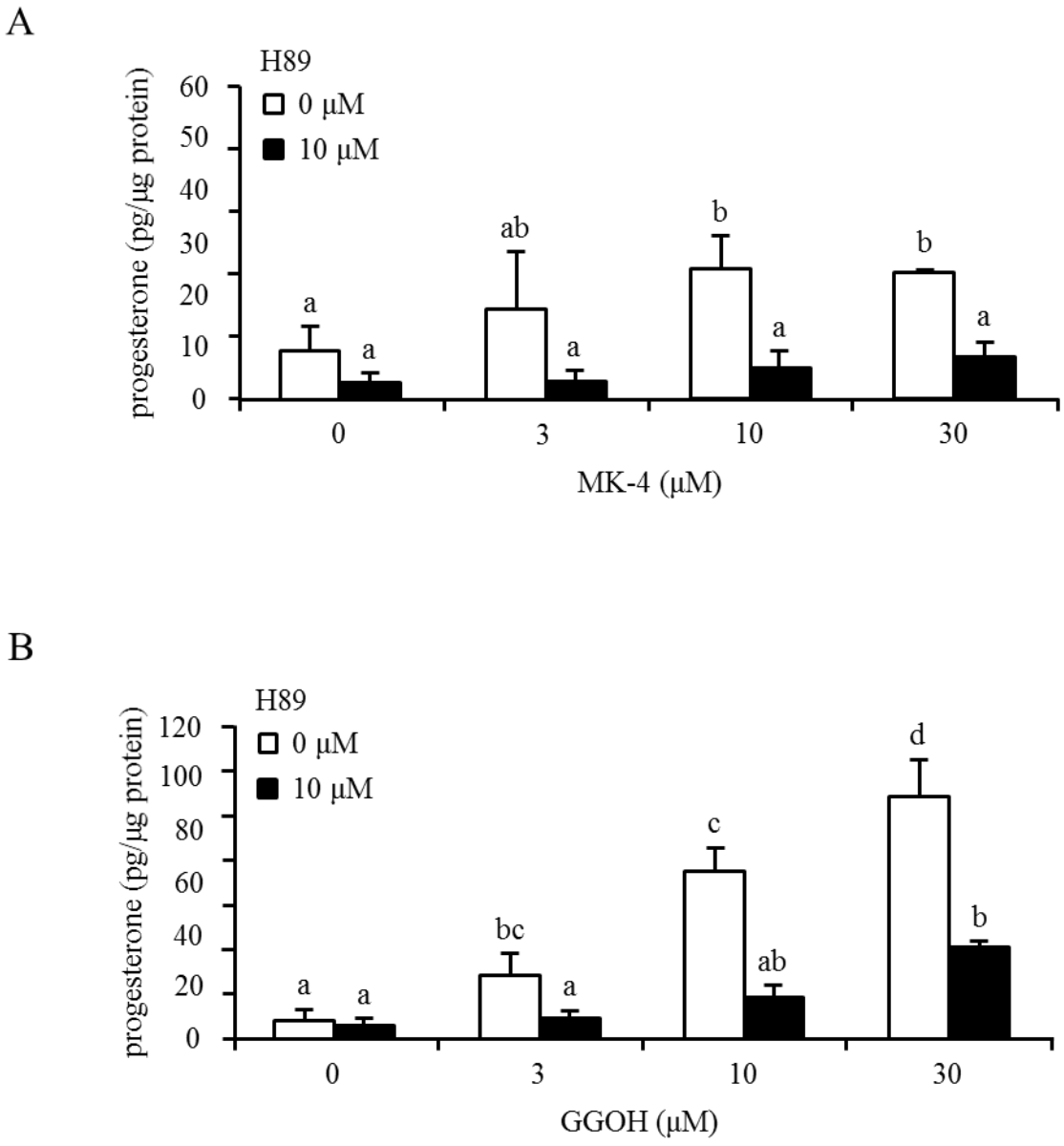


Fig. 7. MK-4 and GGOH enhance the activation of PKA in I-10 cells. I-10 cells were treated with H89 and MK-4 (A) or GGOH (B) for 3 h, and progesterone levels in the culture medium were measured by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

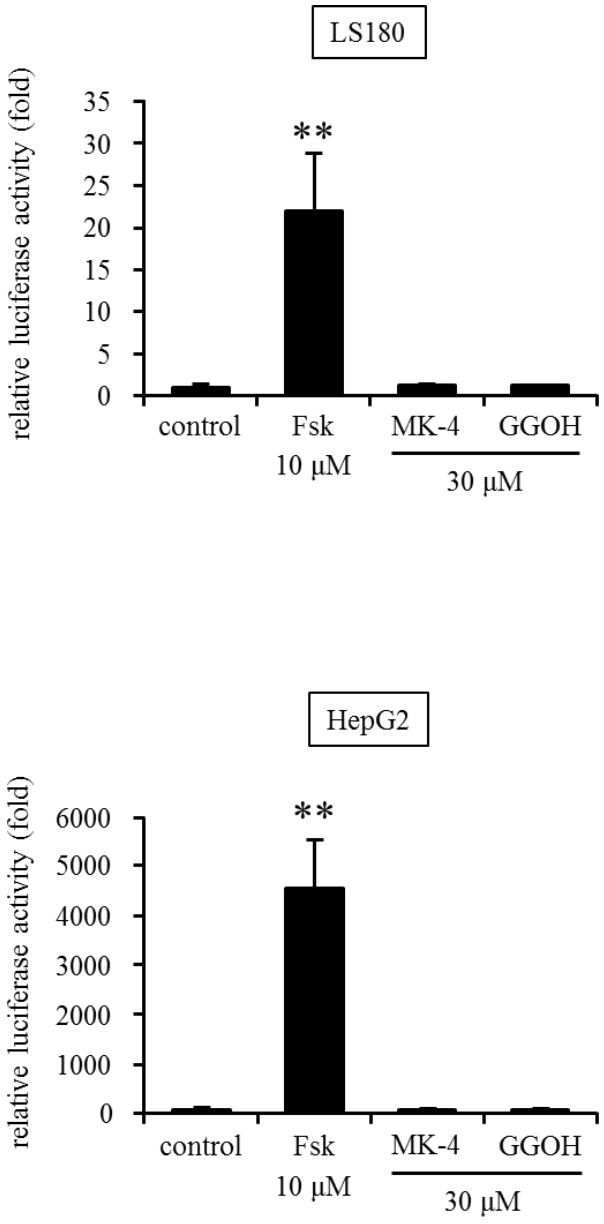


Fig. 8. Effects of MK-4 and GGOH on the luciferase activity in LS180 and HepG2 cells. Cells were transfected with a CRE-inducible reporter gene and then treated with MK-4 or GGOH for 3 h. Reporter activity in cell lysates was measured with the luciferase assay. **P < 0.01.

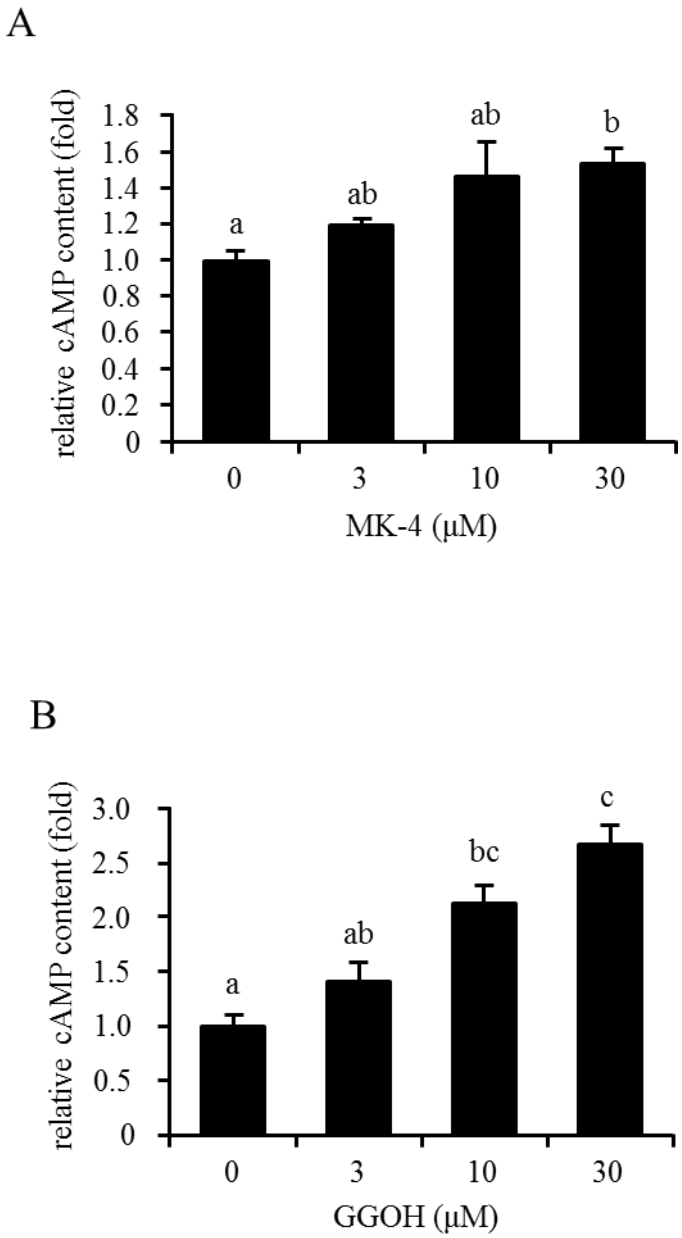


Fig. 9. MK-4 and GGOH stimulates intracellular cAMP levels in I-10 cells. Cells were treated with indicated concentrations of MK-4 (A) or GGOH (B) for 1.5 h, then collected and acetylated before measuring of cAMP levels in cell lysates by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

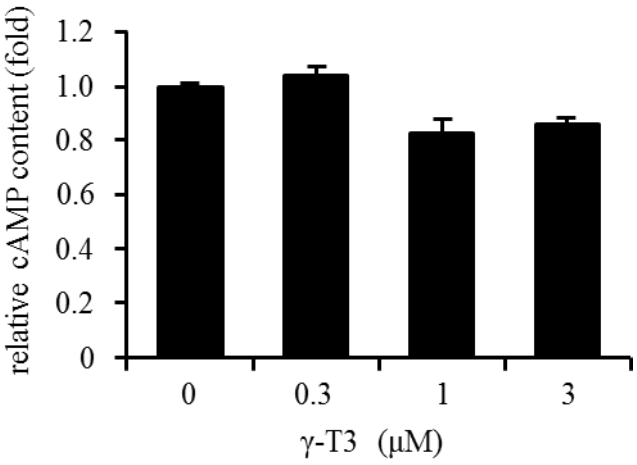


Fig. 10. γ-T3 did not stimulate intracellular cAMP levels in I-10 cells. Cells were treated with indicated concentrations of γ-T3 for 1.5 h, then collected and acetylated before measuring of cAMP levels in cell lysates by EIA. Data are presented as mean ± SD (n = 3).

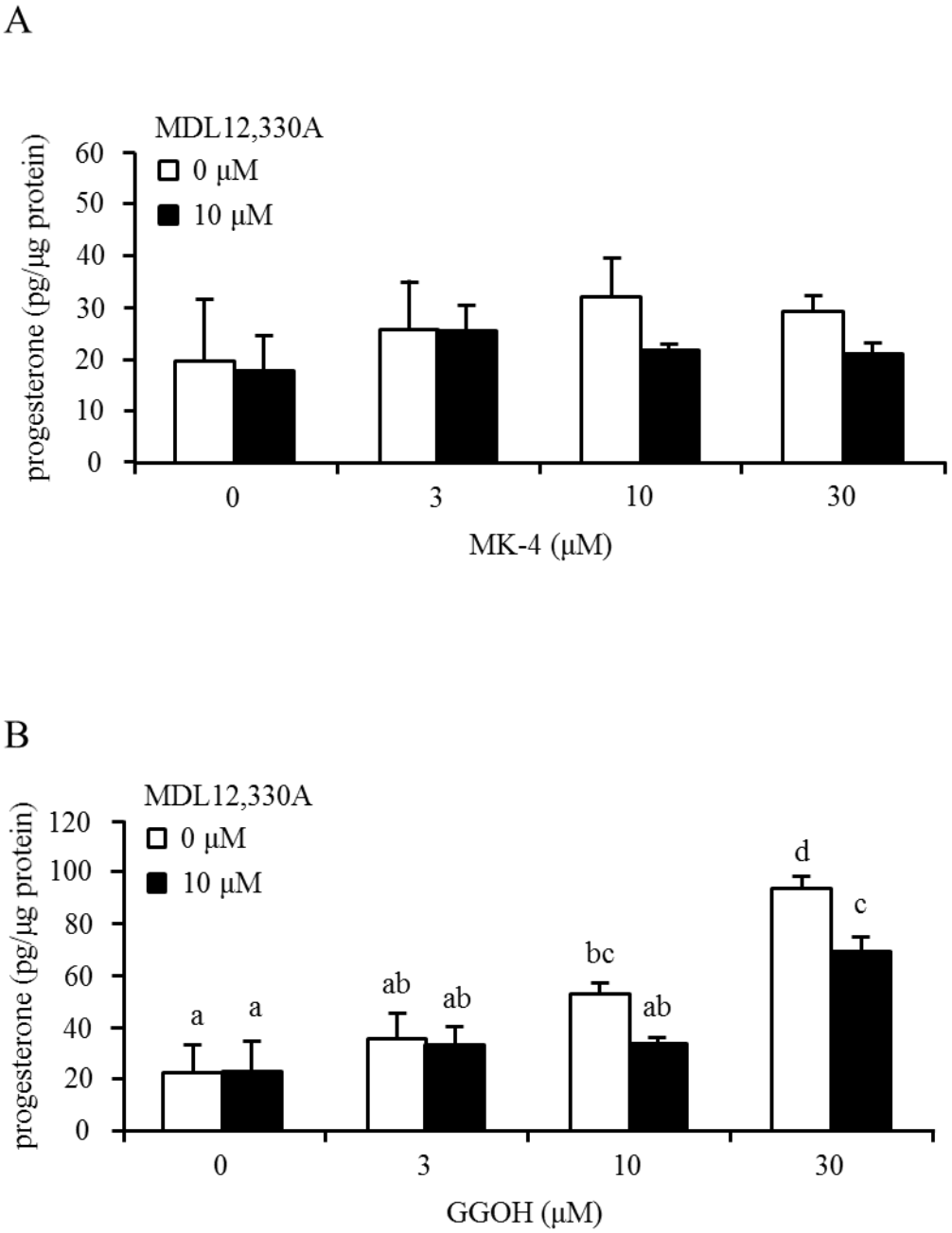


Fig. 11. MK-4 and GGOH enhance the activation of AC in I-10 cells. Cells were treated with MDL12,330A and GGOH (A) or MK-4 (B) for 3 h, and progesterone levels in the culture medium were measured by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

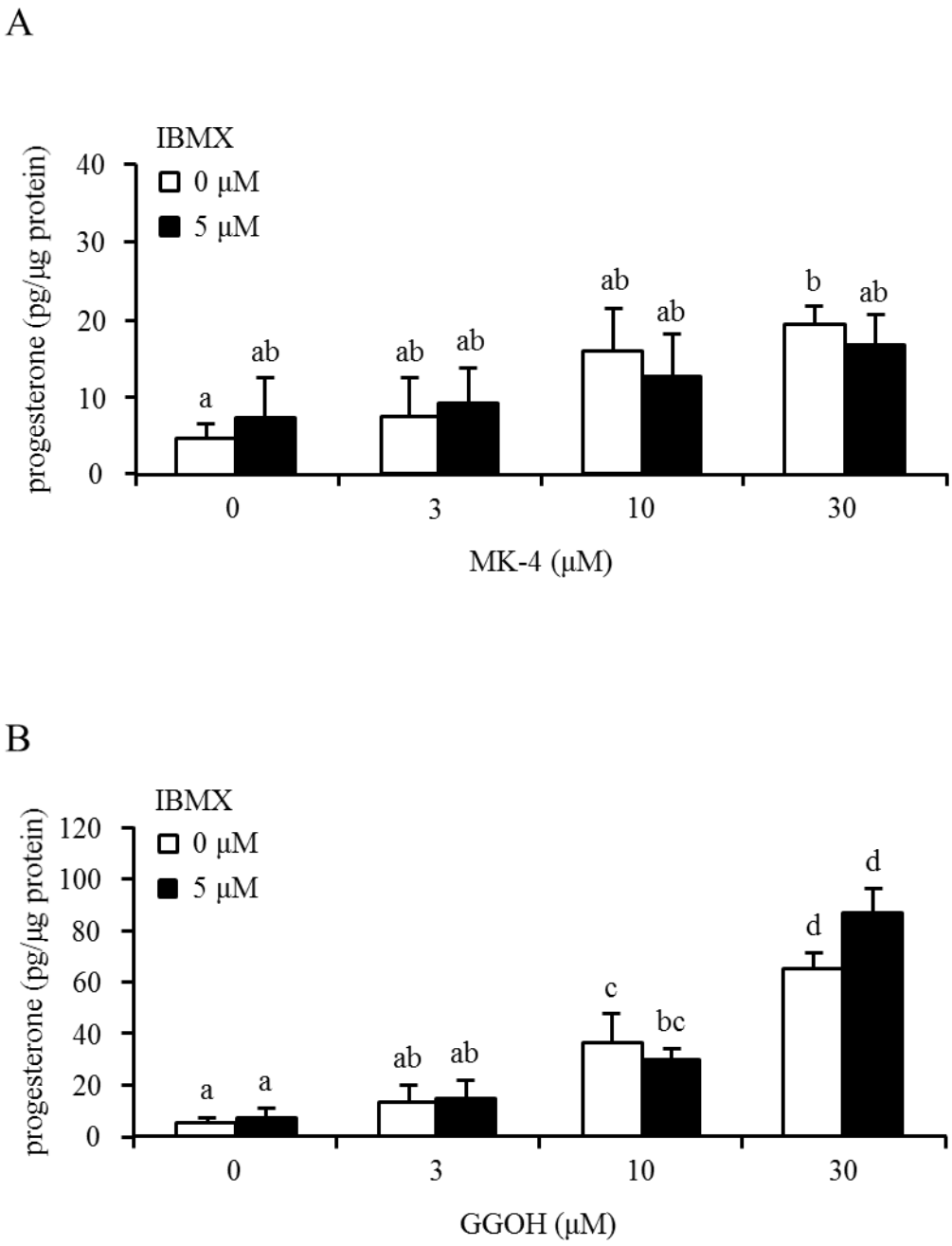


Fig. 12. MK-4 and GGOH induce progesterone production did not regulate PDE in I-10 cells. Cells were treated with IBMX and MK-4 (A) or GGOH (B) for 3 h, and progesterone levels in the culture medium were measured by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

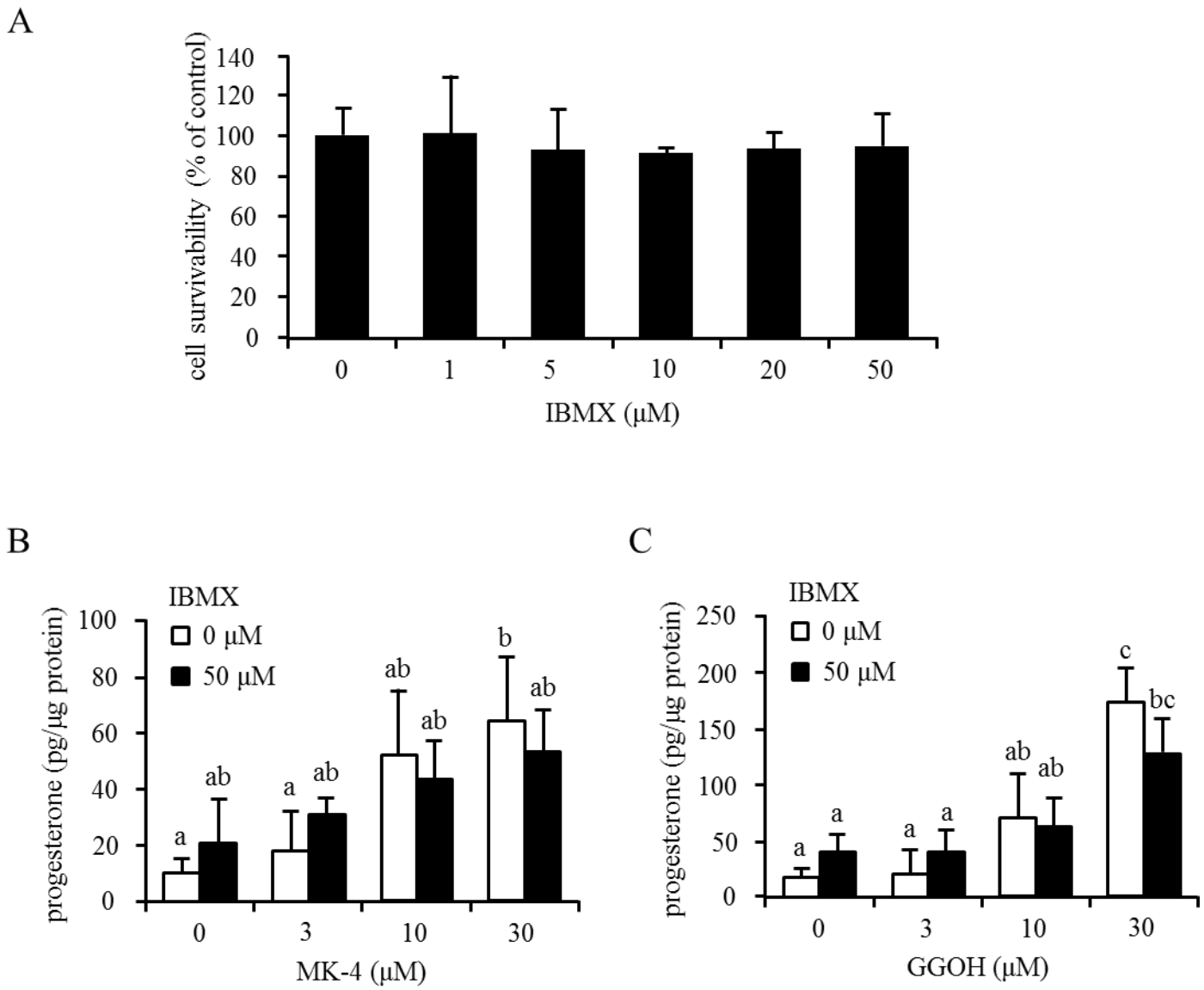
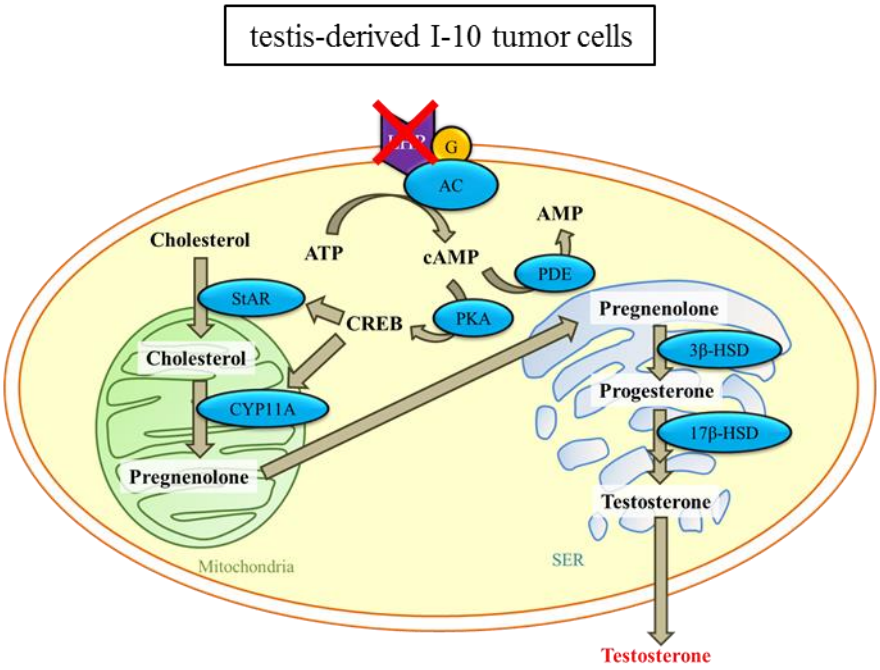


Fig. 13. MK-4 and GGOH did not regulate PDE activity in I-10 cells. (A) Effect of IBMX on cytotoxicity in I-10 cells. Cells were treated with indicated concentrations of MK-4 or GGOH for 24 h. Cells survivability were measured by WST-1. Data are presented as mean ± SD (n=6). Progesterone levels of I-10 cells after 50 μM IBMX and MK-4 (B) or GGOH (C) treatments. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

A



B

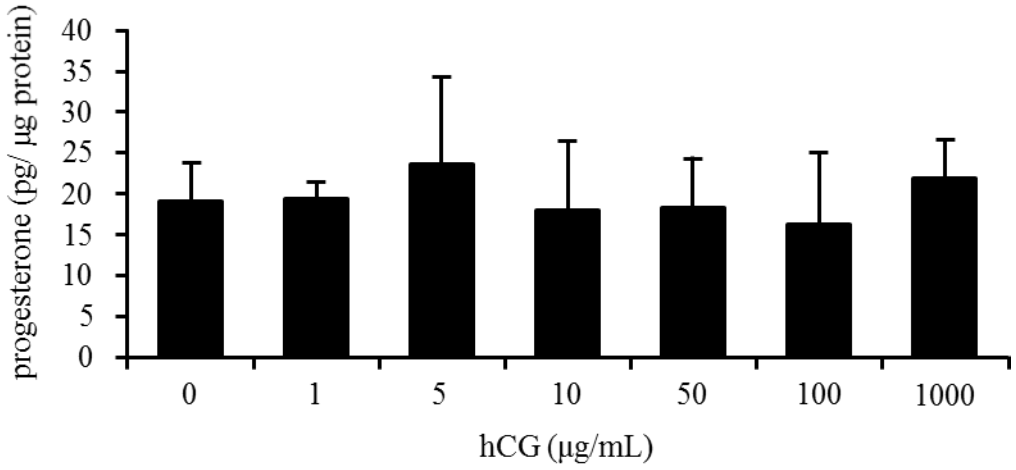


Fig. 14. MK-4 and GGOH stimulate steroidogenesis not through hCG/LH receptor in I-10 cells. (A) Steroidogenesis pathway in I-10 cells. (B) Effects of hCG on progesterone production in I-10 cells. Cells were treated with indicated concentrations of hCG for 24 h. Progesterone levels in the culture medium were measured by EIA. Data are presented as mean ± SD (n = 3).

Chapter 2 MK-4 and GGOH induce the upregulation of mRNA and protein levels of steroidogenesis- related genes in I-10 cells

According to the results of chapter 1, it revealed that MK-4 and GGOH enhance testosterone and progesterone productions via cAMP/PKA signaling pathway in I-10 cells. To further clarify the effects of MK-4 and GGOH on steroidogenesis-related genes, here we measured the mRNA and protein levels of steroidogenesis-related genes in I-10 cells.

Section 1 Effects of MK-4 and GGOH on mRNA levels of steroidogenesis-related genes

MK-4 and GGOH showed similar effects on steroidogenesis in I-10 cells. Here further analyzed the mRNA levels which relate to steroidogenesis.

Experimental procedures

Cell line and culture conditions

As shown in chapter 1, section 1.

Extraction of total RNA

I-10 cells were seeded in 35-mm dishes and incubated overnight. Total RNA was extracted from I-10 cells treated with different concentrations of GGOH for 3 h by using the Isogen acid phenol–guanidine thiocyanate-based reagent (Nippon Gene, Tokyo, Japan). 500 μl Isogen reagent were added and then pipetting. 100 μl chloroform was added and mixed by a vortex mixer for 15 sec. . After storing for 2~3 min at room

temperature, centrifuged at 13,000 rpm for 15 min, an aqueous phase in the top layer were collected. Isopropanol (equal volume of samples) was added and mixed, then stored for 10 min at room temperature. After that, centrifuged at 13,000 rpm for 10 min, samples were separated by decantation. Samples were then washed by 75% ethanol and centrifuged at 13,000 rpm for 5 min twice. After decantation of samples, RNA precipitates were air dried for 15 min. Thus obtained RNA was dissolved in DEPC-treaated dH_2O and then pipetting. quantity and quality of RNA were determined by measuring the absorbency at 260 nm/280 nm wavelength.

cDNA synthesis

For cDNA synthesis, 4 μg of RNA was used as a template. The RNA were added dH_2O containing 10 mM dNTP and oligo$(dT)_{20}$ incubated at 65°C for 5 min by using Thermal cycler (TaKaRa). Incubated the tubes on ice more than 1 min, added 5×RT buffer, 0.1 M DTT, RNaseOUT and Superscript III, spin down briefly and then incubated at 50°C for 60 min and 70°C for 15 min to synthesize cDNA. Synthesized cDNA were diluted 10 fold by dH_2O and stored at -20°C.

Quantitative reverse transcriptase-mediated PCR (qRT-PCR)

cDNA were diluted 30 fold to measure. PCR were carried out on an ABI 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix EX Taq (Takara Bio, Otsu, Japan). Primer sequences are shown in Table 1.

Results and discussion

To further clarify the mechanism by which MK-4 and GGOH induce

steroidogenesis via the cAMP/PKA axis, the mRNA expression of steroidogenesis-related genes was measured in this section. We first estimated *Ac1-Ac10*, *Cyp11a*, and *StAR* mRNA expressions after 1 or 3 h of MK-4 and GGOH treatments (Fig. 15-1~4). However, we only found that the expression of *Ac9* increased following MK-4 treatment for 3 h (Fig. 15-3), and *StAR* mRNA expression increased after 3 h of GGOH treatment (Fig. 15-4). There was no significant change in *Cyp11a* mRNA expression (Fig. 13-4). According to these results, we found that MK-4 and GGOH might regulate cAMP/PKA signaling pathway via modulate different genes which are related to steroidogenesis in I-10 cells.

Table 1. Nucleotide sequences of primers used for qRT-PCR

Gene name	Primer ID	Forward	Reverse
EF1α1	Shi-120, Shi-121	5’-GATGGCCCCAAATTCTTGAAG-3’	5’-GGACCATGTCAACAATGGCAG-3’
Ac1	kinyou001, kinyou002	5’-GGTCCAGTGTTTTCCAGGGT-3’	5’-CACCACACAGCCTTGAGCTA-3’
Ac2	kinyou019, kinyou020	5’-TCAACCCCAAGGGAGAAAGAC-3’	5’-CCATCCAGAGTGTGTCGAGG-3’
Ac3	kinyou005, kinyou006	5’-GGAAAAGGACTCTCCTATGGTGG-3’	5’-GCCTGCTGTCAGTGCCATT-3’
Ac4	kinyou007, kinyou008	5’-ATTGCTGCGTGTTGGGTTTC-3’	5’-CACCAGCCACAGCAGAAGTA-3’
Ac5	kinyou021, kinyou022	5’-GAAACAGCTTGTCTCCAATGTCC-3’	5’-ACCTCAGCTGGGTAGTGAGT-3’
Ac6	kinyou009, kinyou010	5’-TTCCTTTGGAAGCAGCTCGG-3’	5’-ATGGCATTGGTGCAGAGGAA-3’
Ac7	kinyou023, kinyou024	5’-TGGTGACCGACACTACATGC-3’	5’-TGGTGCCGCTTGACATAGAG-3’
Ac8	kinyou013, kinyou014	5’-TCATGATCGCCATCTACGCC-3’	5’-TCCCCAGGAAATCTTCTCCAC-3’
Ac9	kinyou015, kinyou016	5’-CCTGTGTCAGGACAGTTCCATT-3’	5’-TTCTGTGCTGAGTCCAAGGG-3’
Ac10	kinyou017, kinyou018	5’-AGAGCTCGACTCGTACCTGG-3’	5’-CTCTGTGGTGGTCGAGGTTT-3’
Cyp11a	Mine-021, Mine-022	5’-CGTGACCTTGCAGAGGTACACT-3’	5’-GCTGGAATCTTGTAATTACGAAGCA-3’
StAR	kinyou025, kinyou026	5’-GGAGCTCTCTGCTTGGTTCTC-3’	5’-TTAGCACTTCGTCCCCGTTC-3’

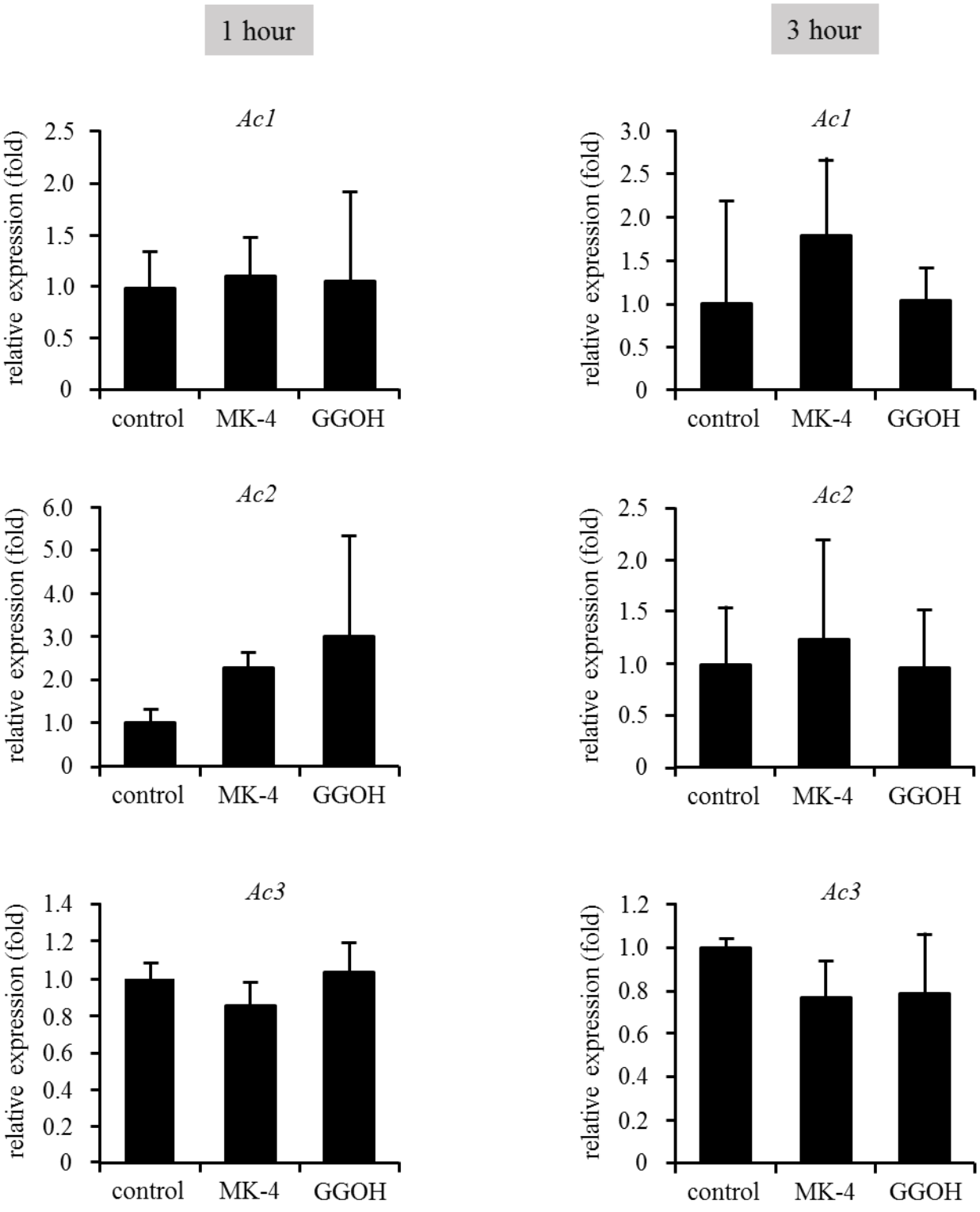


Fig. 15-1. MK-4 and GGOH regulate steroidogenesis-related genes mRNA levels in I-10 cells. I-10 was incubated with treatments of MK-4 or GGOH for 1 (left) or 3 (right) h; mRNA levels of steroidogenesis-related genes (*Ac 1-10*, *Cyp11a*, and *StAR*) were then measured by qRT-PCR. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

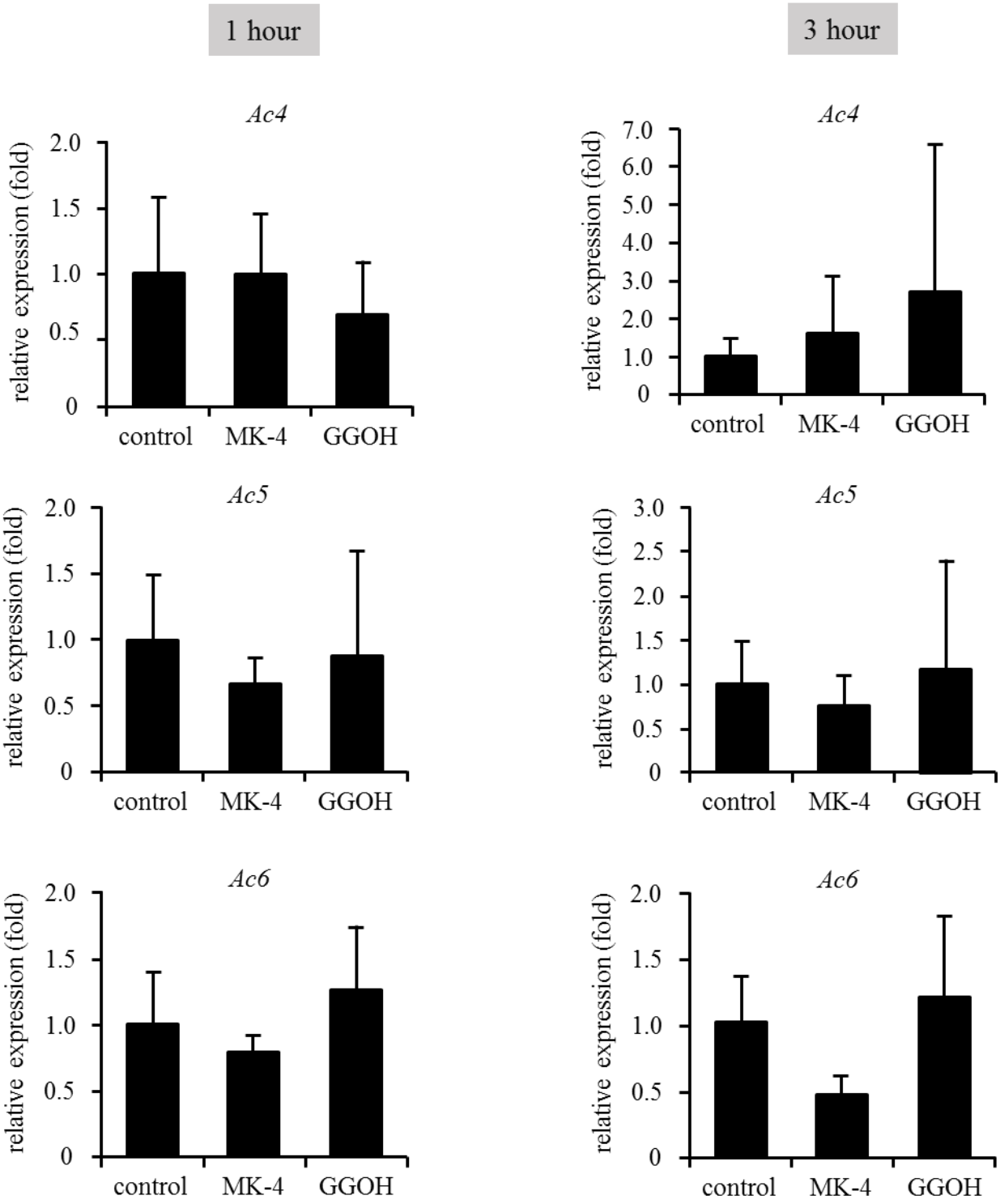


Fig. 15-2. MK-4 and GGOH regulate steroidogenesis-related genes mRNA levels in I-10 cells. I-10 was incubated with treatments of MK-4 or GGOH for 1 (left) or 3 (right) h; mRNA levels of steroidogenesis-related genes (*Ac 1-10*, *Cyp11a*, and *StAR*) were then measured by qRT-PCR. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

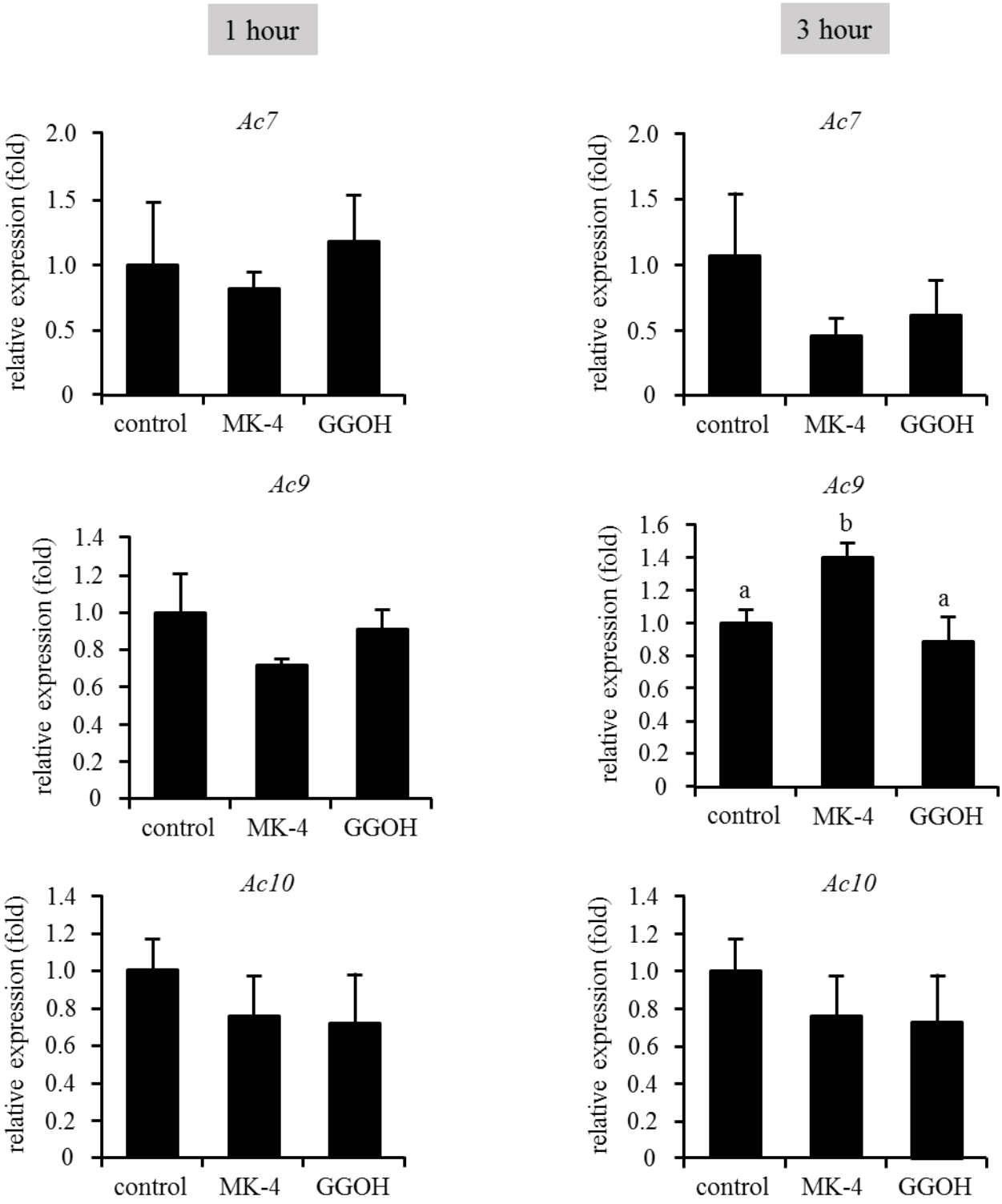


Fig. 15-3. MK-4 and GGOH regulate steroidogenesis-related genes mRNA levels in I-10 cells. I-10 was incubated with treatments of MK-4 or GGOH for 1 (left) or 3 (right) h; mRNA levels of steroidogenesis-related genes (*Ac 1-10*, *Cyp11a*, and *StAR*) were then measured by qRT-PCR. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

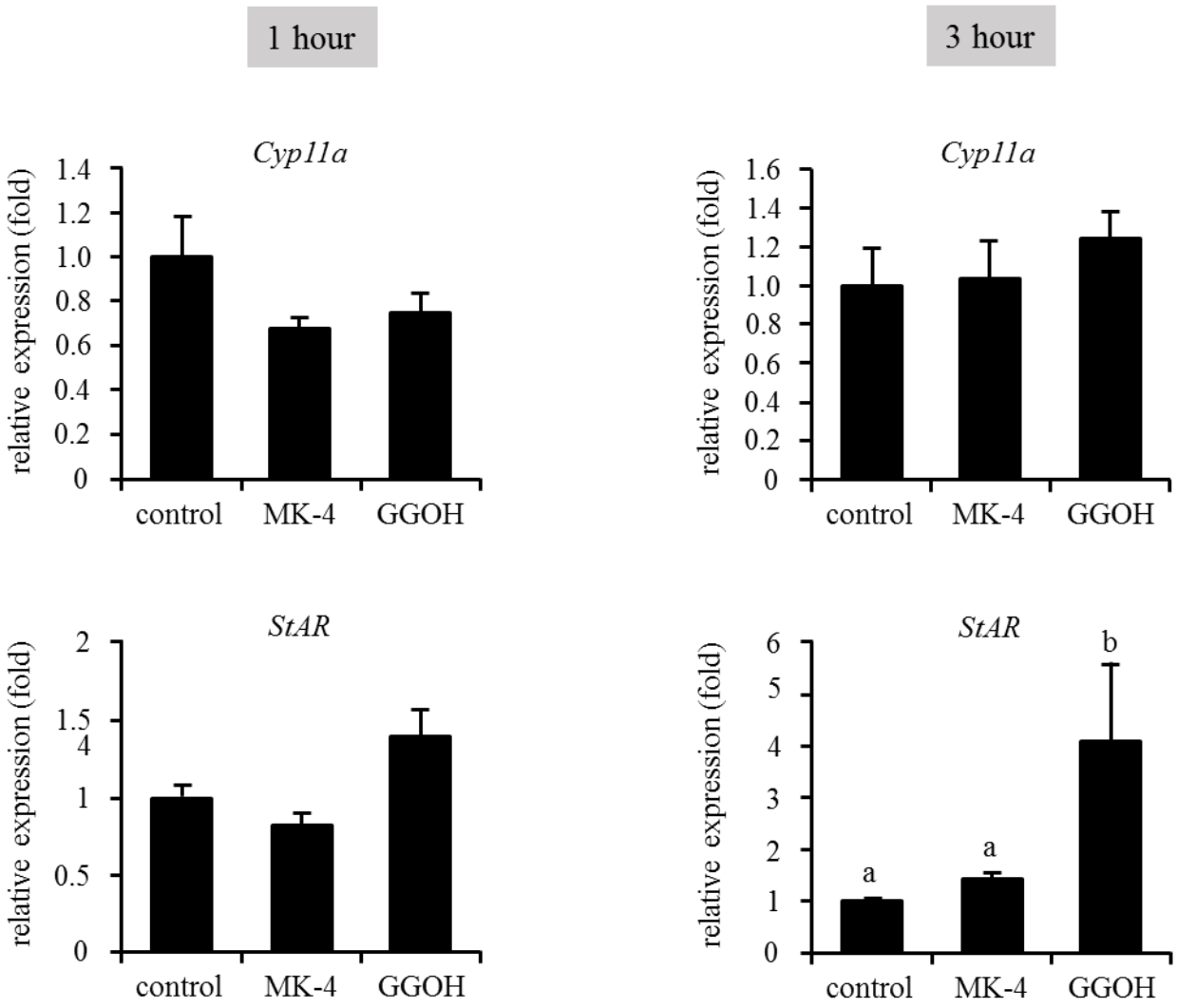


Fig. 15-4. MK-4 and GGOH regulate steroidogenesis-related genes mRNA levels in I-10 cells. I-10 was incubated with treatments of MK-4 or GGOH for 1 (left) or 3 (right) h; mRNA levels of steroidogenesis-related genes (*Ac 1-10*, *Cyp11a*, and *StAR*) were then measured by qRT-PCR. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

Section 2 Effects of MK-4 and GGOH on protein levels of steroidogenesis-related genes

According to the results in previous section, although testosterone and progesterone productions were stimulated by the presence of MK-4 and GGOH, MK-4 and GGOH modulated different RNA expression in I-10 cells. We also investigated the effects of MK-4 and GGOH on protein levels.

Experimental procedures

Cell line and culture conditions

As shown in chapter 1, section 1.

Western blot analysis

I-10 cells were seeded in 10-cm dishes and incubated overnight. The culture medium was refreshed with MK-4 or GGOH-containing medium, followed by incubation for 3, 6 or 24 h. Cells were harvested by scraping, and total cellular protein was prepared in lysis buffer consisting of 50 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.1% SDS, 5 mM EDTA, Complete proteinase inhibitor cocktail, and PhosSTOP phosphatase inhibitor cocktail (both from Roche Applied Science, Mannheim, Germany). Protein concentrations were measured by the Lowry method. Cell extracts (15 μg protein) were resolved by electrophoresis on a 10–20% SDS polyacrylamide gel (Wako Pure Chemical Industries, Osaka, Japan) at 150 V for 150 min. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), which was blocked overnight with blocking buffer containing 10 mM Tris-HCl (pH 7.5),

150 mM NaCl, 0.1% Tween 20, and 5% (w/v) skim milk. The membrane was then incubated with buffer (Can Get Signal; Toyobo Co., Tokyo, Japan) containing an antibody against StAR (Affinity BioReagents, Golden, CO, USA) or Cyp11a (Millipore) overnight, followed by incubation with horseradish peroxidase-linked secondary antibody for 1 h at room temperature. Immunoreactive bands were detected with Immobilon Western Detection Reagent (Millipore) on an LAS-4000 mini luminescent image analyzer (Fujifilm, Tokyo, Japan). Relative expression levels of each protein were normalized to the amount of α-tubulin detected using a specific antibody (Sigma-Aldrich).

Lowry method for protein quantitation

As shown in chapter 1, section 1.

Statistical analysis

As shown in chapter 1, section 1.

Results and discussion

As for the protein levels, it is consistent with the effect of GGOH on *StAR* mRNA level, GGOH increased StAR protein levels at 3, 6 and 24 h. However MK-4 showed a tendency to induce StAR at 6 and 24 h as shown in Fig. 16. Cyp11a protein level was significantly increased after GGOH treatment at 3 h but did not change at 6 or 24 h (Fig. 17). Refer to section 1 in this chapter, these results indicated that MK-4 and GGOH induce the upregulation of certain steroidogenesis-related genes expression via the cAMP/PKA signaling pathway to enhance steroid production but not the same.

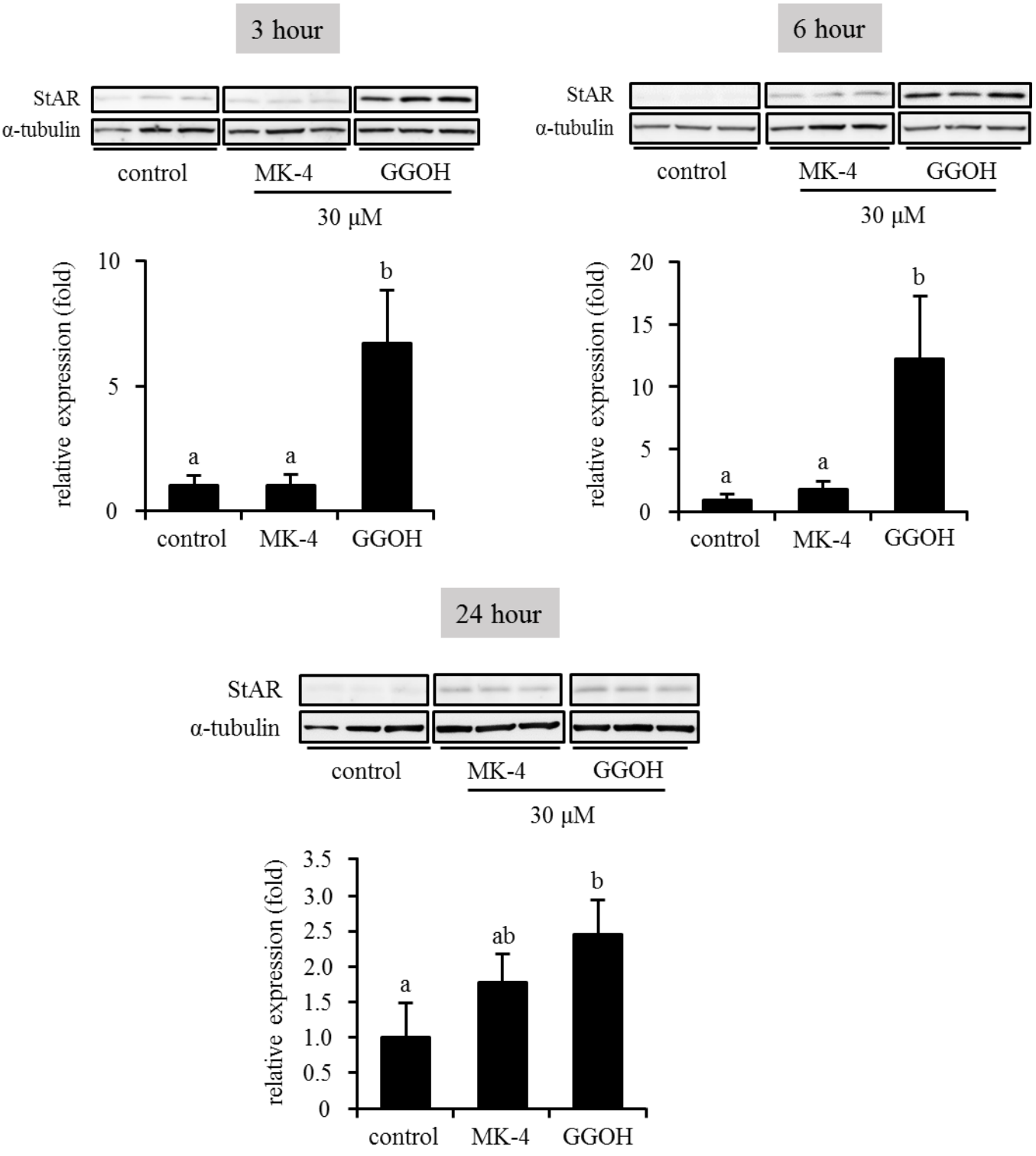


Fig. 16. MK-4 and GGOH regulate steroidogenesis-related genes protein levels in I-10 cells. Cells were treated with GGOH for 3, 6 or 24 h, and protein levels of StAR were measured by western blotting. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

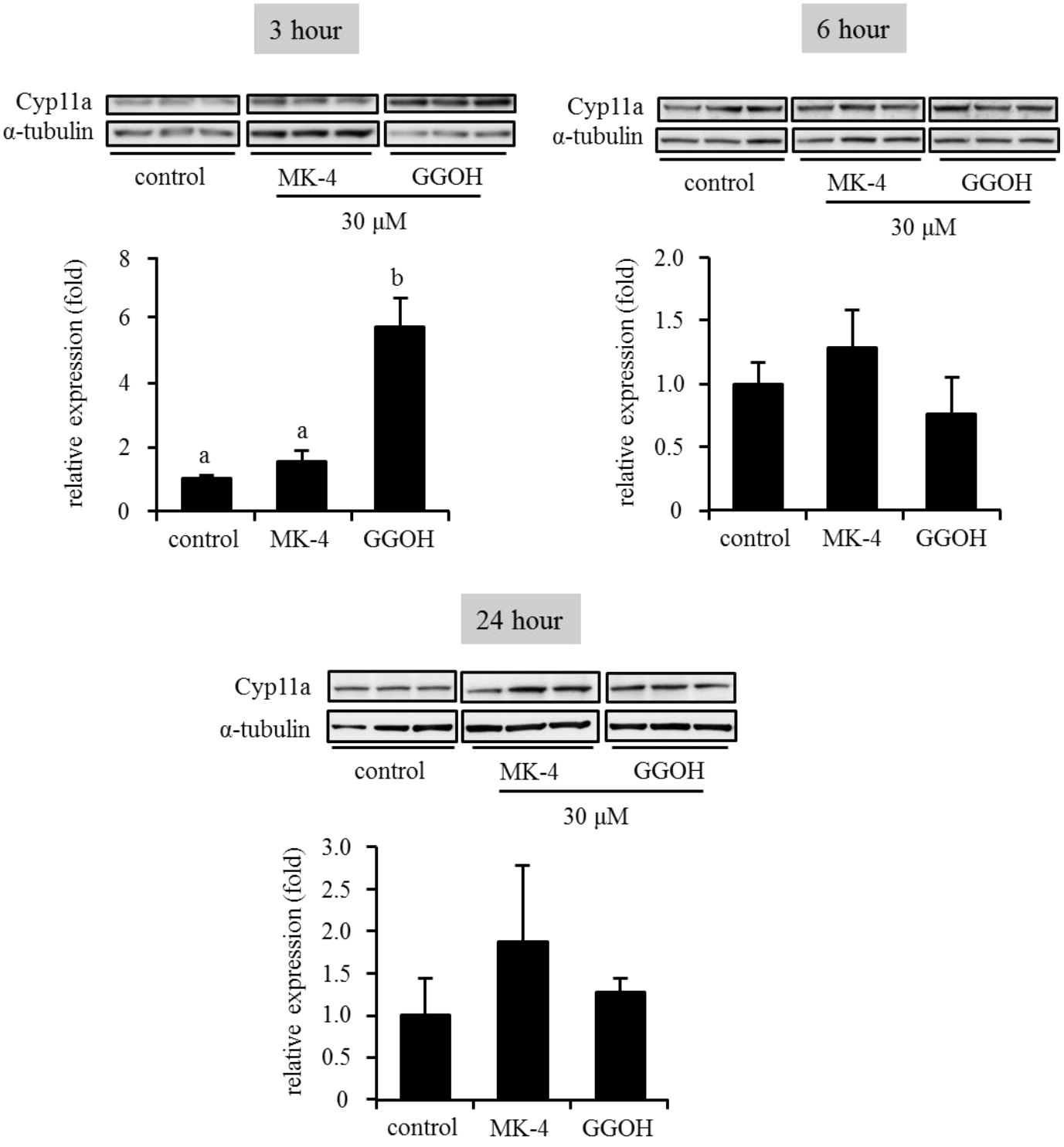


Fig. 17. MK-4 and GGOH regulate steroidogenesis-related genes protein levels in I-10 cells. Cells were treated with GGOH for 3, 6 or 24 h, and protein levels of Cyp11a were measured by western blotting. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

Section 3 Effects of MK-4 and GGOH on progesterone productions after adenylate cyclase mRNA knockdown

Results of section 1 and 2 in this chapter showed that MK-4 and GGOH enhanced testosterone and progesterone productions via stimulated different mRNA or protein expressions of steroidogenesis-related genes. We further knockdown the AC expression by using siRNA to examine their effects on AC activation.

Experimental procedures

Cell line and culture conditions

As shown in chapter 1, section 1.

RNA interference

Stealth RNAi short interfering RNA (siRNA) for mouse *Ac9* (Invitrogen) was used for gene knockdown experiments. The double-stranded siRNA sequence was as follows: 5'-CAU AGG AGU AGA AGA GGC CAG UGA A-3'. The negative control siRNA was also purchased from Invitrogen. Cells were seeded in a 6-well plate and transfected with 10 μM siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions.

Statistical analysis

As shown in chapter 1, section 1.

Results and discussion

To confirm that MK-4 and GGOH stimulate steroidogenesis via induction of AC activity, we knocked down *Ac3* and *Ac9* expression by using siRNA. Our previous study found that the *Ac9* isoform mRNA was highly expressed in I-10 cells and *Ac3* isoform expression was enhanced following forskolin (as AC1-8 activator) treatment. In Fig. 18, the effect of Ac9 and Ac3 knockdown on progesterone production in I-10 cells was shown. The cells were added with treatments of forskolin, MK-4 and GGOH for 3 h after siRNA transfection. In Ac9-deficient cells, GGOH treatment had no effect on progesterone levels relative to the control group that forskolin still enhanced progesterone levels in Ac9-deficient cells (Fig. 18A), and it is consistent with the reports that forskolin only activate Ac1-8 but not Ac9. In addition, by using Ac3-deficient cells, the enhancement of progesterone level by forskolin and GGOH were abolished (Fig 18B). However, we did not find the evidence of MK-4 on Ac3 or Ac9 activation here. These results indicate that GGOH, but not MK-4, stimulates steroidogenesis via regulation of Ac activity.

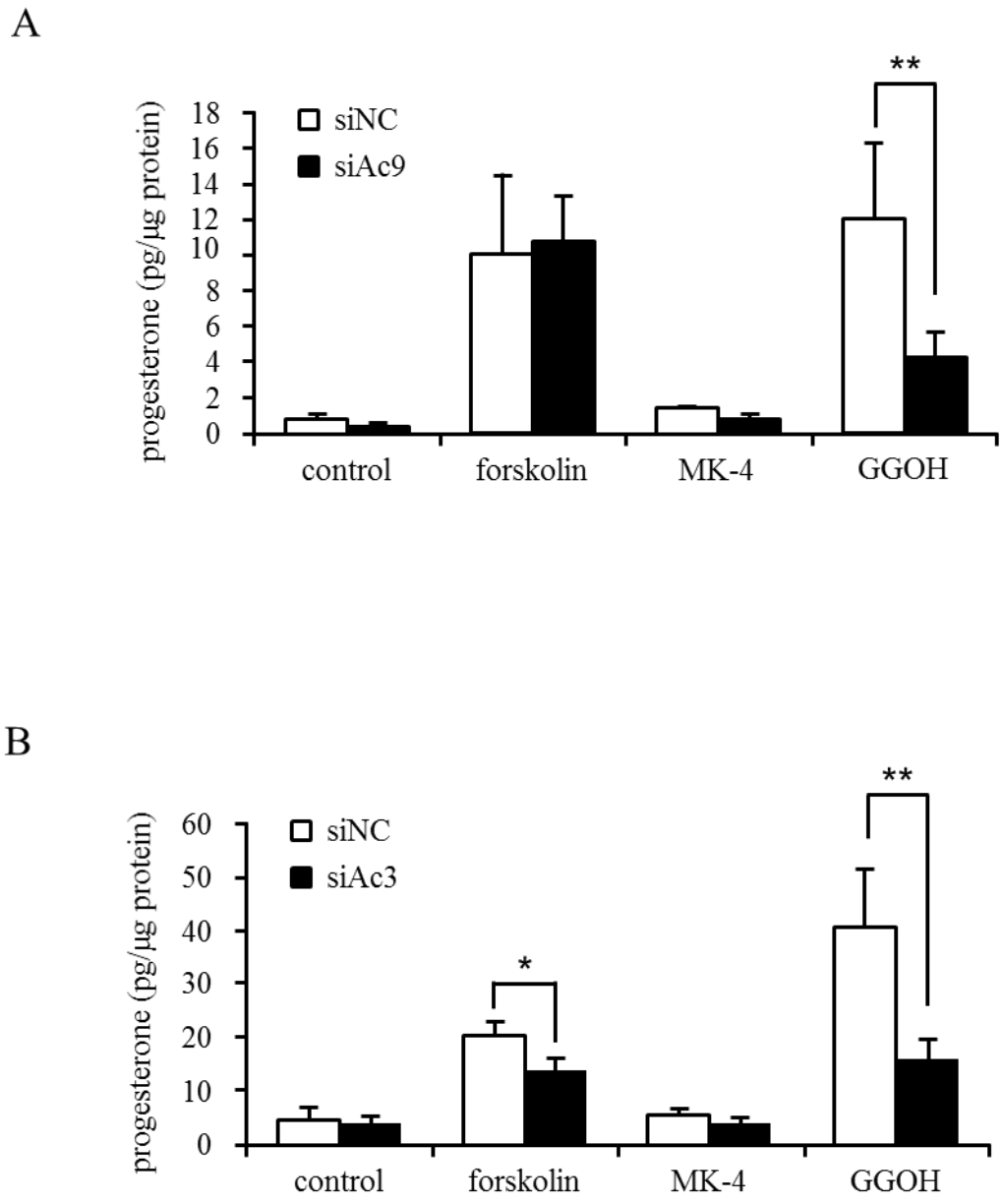


Fig. 18. Effects of Ac9 and Ac3 knockdown on progesterone production in I-10 cells. I-10 cells were transfected with siRNAs targeting Ac9 (A) and Ac3 (B), and progesterone levels in the culture medium were measured by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

Chapter 3 MK-4 but not GGOH promotes glucose-stimulated insulin secretion in INS-1 cells

Previous study showed that MK-4 and GGOH induced insulin secretion in a dose- and time-dependent manner after stimulated with glucose in cultured mouse pancreatic islets. Therefore, the present study investigated the effect of MK-4 and GGOH on glucose-stimulated insulin secretion (GSIS), then further clarified the mechanism of MK-4 and GGOH modulating insulin secretion in rat insulinoma INS-1 cells.

Section 1 Effects of MK-4 and GGOH on GSIS in INS-1 cells

To explore a novel action of MK-4 and GGOH on GSIS, we used a rat insulinoma cell line which exists GSIS response. We firstly investigated whether MK-4 and GGOH mediate the amplification of GSIS or not here.

Experimental procedures

Cell line and culture conditions

Cell line: INS-1 rat insulinoma cell line was a kind gift from Dr. Harada, Osaka Prefecture University.

Culture medium: INS-1 cells were maintained in RPMI1640 medium (Sigma-Aldrich) supplemented with 11.1 mM glucose sodium pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol, 10% fetal bovine serum (Biosera, Boussens, France), 50 U/ml penicillin, and 50 μg/ml streptomycin.

Culture conditions: INS-1 cells were cultured in a 5% CO_2 humidified incubator at 37°C.

Cell passage: cells were used between passages 21- 30. Cells were cultured in a 10 cm dish, removed the culture medium by using aspirator, then washed by PBS for twice. 0.25% Trypsin-EDTA were added and incubated for 5 min, then added fresh medium, carefully resuspend cells. Dilute cells with fresh medium in new dish were then placed back to the incubator. For experiments, cells were used when they reached 60–70% confluence.

Cell proliferation assay

As shown in chapter 1 section 1.

Glucose-stimulated insulin secretion (GSIS)

INS-1 cells were seeded into 24-well plate at a density of 0.5×10^4 cells/well and incubated overnight. The medium was removed the following day, KRBH buffer with 2.8 mM glucose were added into well pre-incubated for 1 h, then the KRBH buffer were replaced with 2.8 – 33.6 mM glucose containing 0 – 3 μM MK-4 or 10 μM GGOH for 1 h. The KRBH were centrifuged at 1,000 × g for 5 min. The collected mediums were stored at -20°C before use. Insulin concentrations were determined with an insulin EIA kit (Morinaga, Tokyo. Japan). The collected mediums were diluted 10 fold for insulin measurement. Protein concentrations were measured by the Lowry method.

*KRBH (Krebs-Ringer bicarbonate HEPES) buffer containing 135 mM NaCl, 3.6 mM KCl, 0.5mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES and 0.2 % BSA.

Measurement of insulin levels

According to the manufacturer's instructions. 95 μl enzyme-labeled anti-insulin reagents were added into well. The 5 μl standard and 10 fold diluted sample were then added, and incubated overnight. The following day, wells were washed 5 times by wash buffer, then 100 μl enzyme-labeled antibody was added and incubated for 1 h. After incubation with enzyme-labeled antibody, wells were washed 5 times by wash buffer, 100 μl enzyme substrate solution (TMB solution) was added and the samples were protected from light, after incubation for 30 min, 100 μl stop solution was added into the wells which the absorbance were measured within 30 min. Measured absorbance using an ELISA reader at 450 nm with a reference wavelength at 630 nm.

Lowry method for protein quantitation

As shown in chapter 1, section 1.

Statistical analysis

As shown in chapter 1, section 1.

Results and discussion

To determine the dosage of MK-4 and GGOH, we first analyzed the cell survival rate of INS-1 β-cells by using WST-1 assay (Fig. 19), when the concentrations of MK-4 and GGOH under 3 μM and 10 μM did not bring any toxicity effects in INS-1 cells, respectively. The concentrations of MK-4 (3 μM) and GGOH (10 μM) were used for the following experiments.

In Fig. 20, GGOH did not increase the GSIS in INS-1 cells in the case of 2.8 mM

glucose stimulation, whereas 3 μM of MK-4 enhanced insulin secretion even in the basal level of glucose stimulation (2.8 mM). Further experiments showed that 1 μM of MK-4 increased the insulin secretion with glucose dose-dependent manner (Fig. 21).

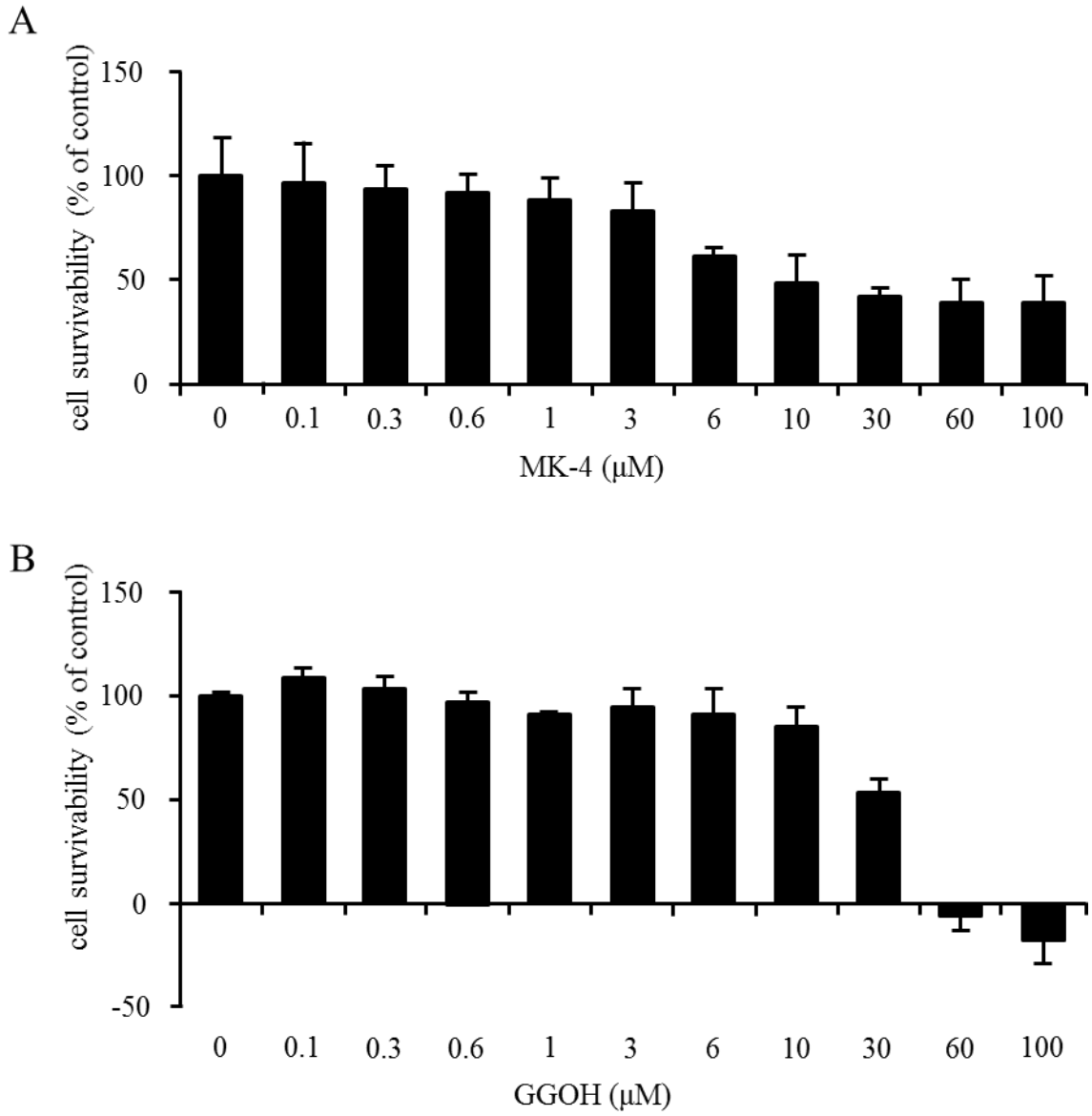


Fig. 19. Effects of MK-4 (A) and GGOH (B) on cytotoxicity in INS-1 cells. Cells were treated with indicated concentrations of MK-4 or GGOH for 24 h. Cells survivability were measured by WST-1. Data are presented as mean ± SD (n=6).

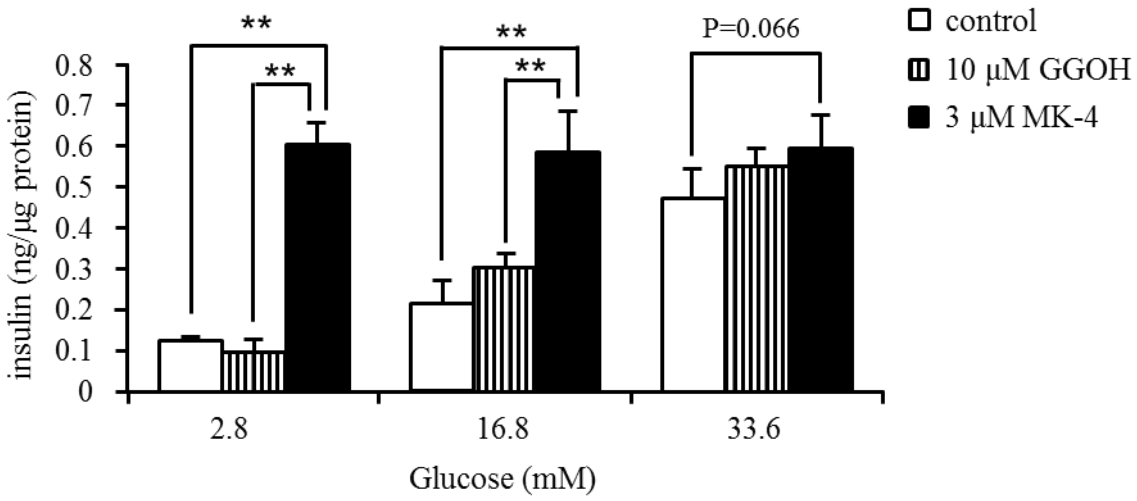


Fig. 20. MK-4 but not GGOH regulate glucose stimulate insulin secretion in INS-1 cells. Cells were treated with indicated concentrations of glucose and MK-4 or GGOH for 1 h. Insulin concentration were measured by EIA. Data are presented as mean ± SD (n=3). **$p < 0.01$.

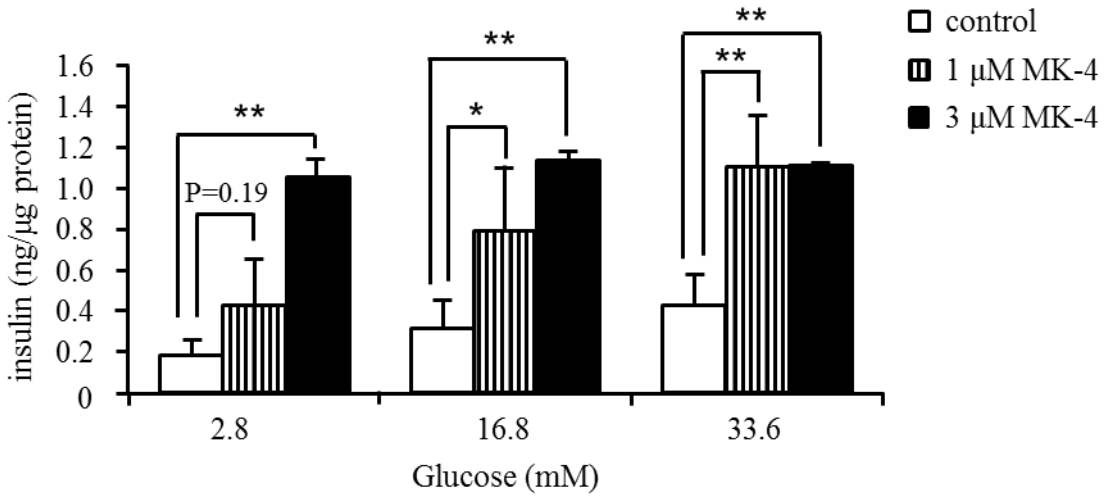


Fig. 21. MK-4 regulates glucose stimulate insulin secretion in INS-1 cells. Cells were treated with indicated concentrations of glucose and MK-4 for 1 h. Insulin concentrations were measured by EIA. Data are presented as mean ± SD (n=3). *p < 0.05, **p < 0.01.

Section 2 Effects of MK-4 and GGOH on cAMP/PKA signaling pathway

According to the previous results, we hypothesized that MK-4 can also regulate glucose-stimulated insulin secretion (GSIS) via modulating cAMP/PKA signaling pathway in pancreatic β-cells.

Experimental procedures

Measurement of cAMP levels

As shown in chapter 1, section 2.

CRE-Reporter gene assay

As shown in chapter 1, section 2.

Extraction of total RNA

As shown in chapter 2, section 1.

cDNA synthesis

As shown in chapter 2, section 1.

Quantitative reverse transcriptase-mediated PCR (qRT-PCR)

As shown in chapter 2, section 1.

Statistical analysis

As shown in chapter 1, section 1.

Results and discussion

To further clarify the role of MK-4 and GGOH on GSIS, the cAMP levels were measured, and 1 and 3 μM of MK-4 enhanced cAMP levels (Fig. 22A), however, cAMP levels did not promoted by the 1-10 μM of GGOH treatment (Fig. 22A and 22B). In addition, through CRE-reporter gene assay, it was found that the luciferase activity in 1-3 μM of MK-4 and 10μM GGOH treatment did not changed (Fig. 23A and 23B). PKA inhibitor H89 treatment also did not affect on MK-4 mediated GSIS (Fig. 24), which showed that MK-4 and GGOH might not regulate PKA activity in INS-1 cells. Finally, we also determined the mRNA levels of *Ac* in INS-1 cells, and found that *Ac6*, *Ac8* and *Ac9* were highly expressed in INS-1 cells, therefore we firstly investigated the effects of MK-4 on the mRNA expression of *Ac*s for 30 and 60 min treatments (Fig. 25). We did not find any significant difference on the mRNA levels of *Ac6*, *Ac8* and *Ac9* after MK-4 treatment in INS-1 cells. Further clarification of the mechanism involved in the effect of MK-4 on GSIS is necessary in INS-1 cells by using other methods in the near future.

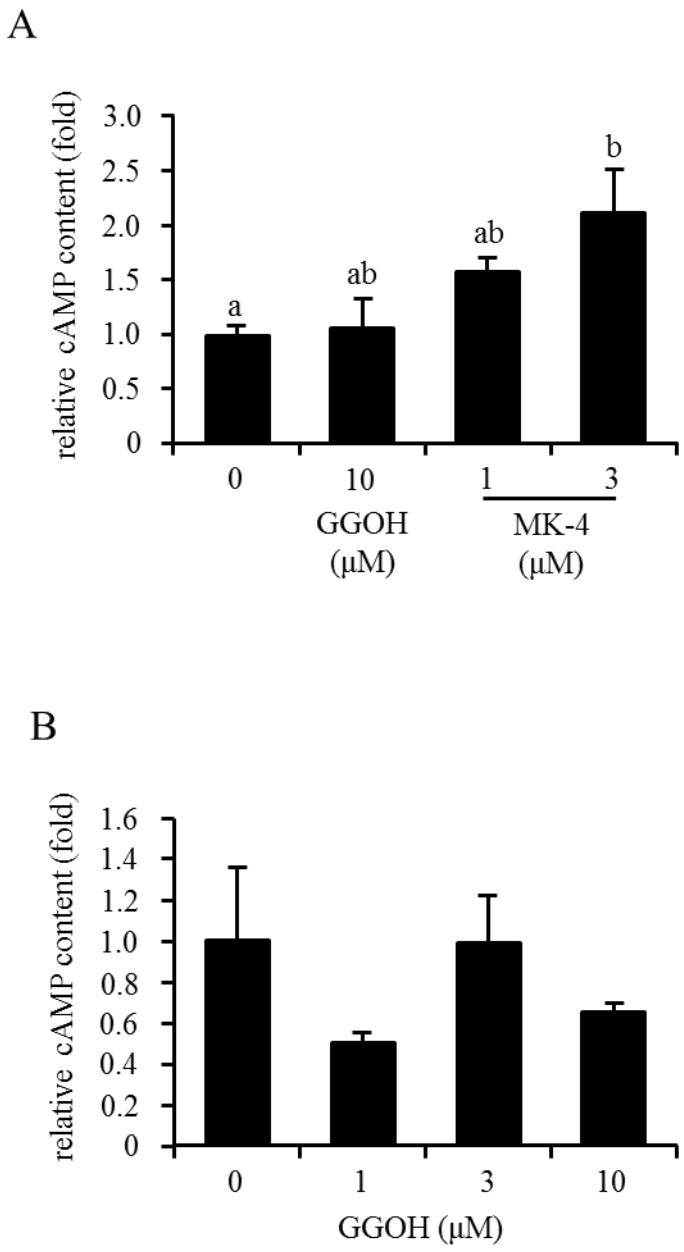


Fig. 22. MK-4 but not GGOH stimulates intracellular cAMP levels in INS-1 cells. Cells were treated with indicated concentrations of MK-4 (A) or GGOH (B) for 1 h, then collected and acetylated before measuring of cAMP levels in cell lysates by EIA. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences ($P < 0.05$).

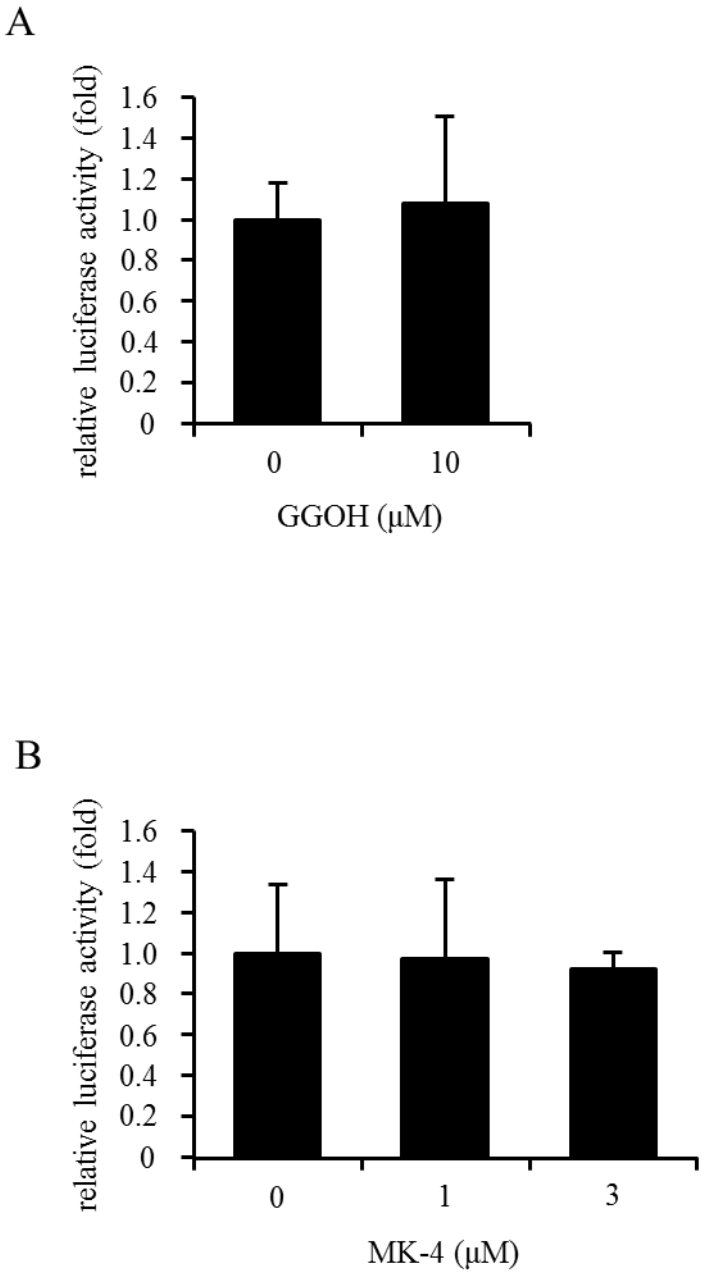


Fig. 23. Effects of MK-4 and GGOH on PKA activity in INS-1 cells. Cells were transfected with a CRE-inducible reporter gene and then treated with MK-4 (A) or GGOH (B) for 3 h. Reporter activity in cell lysates was measured with the luciferase assay. Data are presented as mean ± SD (n = 3).

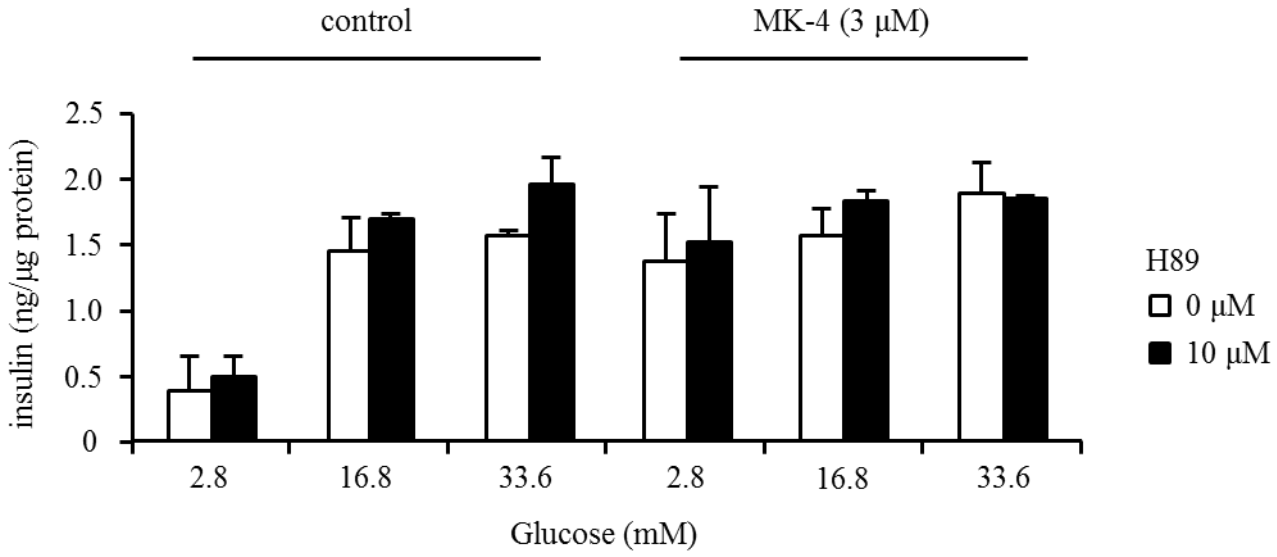


Fig. 24. Effect of MK-4 on activation of PKA in INS-1 cells. Cells were treated with indicated concentrations of glucose and MK-4 with H89 for 1 h. Insulin concentrations were measured by EIA. Data are presented as mean ± SD (n=3).

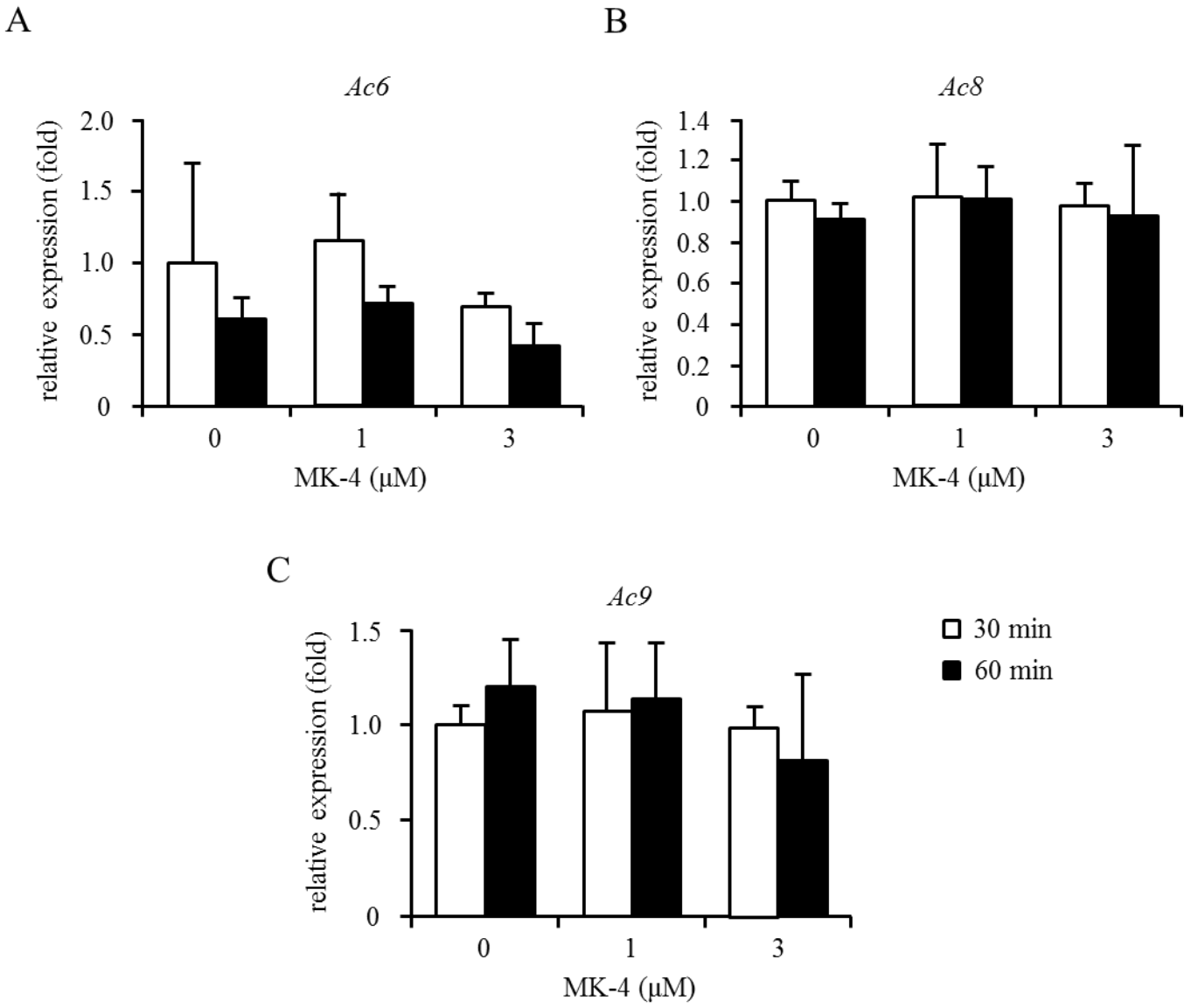


Fig. 25. Effects of MK-4 and on Acs mRNA levels in INS-1 cells. Cells were treated with MK-4 or GGOH for 30 (white) or 60 (black) min; mRNA levels of *Ac6* (A), *Ac8* (B) and *Ac9* (C) genes were then measured by qRT-PCR. Data are presented as mean ± SD (n = 3).

Chapter 4 Conclusion

In the present study, we focused on the effects of MK-4 and GGOH on steroidogenesis and glucose-stimulated insulin secretion. It revealed that MK-4 enhanced not only steroidogenesis but also insulin secretion, and it might be due to regulation of the cAMP/PKA signaling pathway. On the other hand, GGOH markedly enhanced steroidogenesis in I-10 cells, but there is no change on GSIS in INS-1 cells. It is interesting that one study reported by Ichikawa et al. (13) found that MK-4 but not GGOH or other VK enhance the activation of PKA in osteoblastic cells, it showed that MK-4 and GGOH might enhance PKA activity in different cell lines.

Functions of MK-4 and GGOH on steroidogenesis in I-10 cells

Here we firstly elucidated that MK-4 and GGOH have similar functions on steroidogenesis. Although both MK-4 and GGOH regulate cAMP/PKA signaling pathway but they act in different mRNA, proteins and enzymes. GGOH seems possess stronger steroidogenesis ability than MK-4 in I-10 cells. As mentioned in section 2 of chapter 1, I-10 cells lack the LH/hCG receptor that MK-4 and GGOH cannot binding to the receptor to stimulate steroidogenesis, the mechanism of MK-4 and GGOH in I-10 cells should be conducted in the near future.

Role of MK-4 on GSIS in INS-1 cells

Recent studies of insulin secretion in islet which are focus on glucagon-like peptide-1 (GLP-1) receptor-mediated pathway in the pancreatic β-cells. The novel function of MK-4 on GSIS was summarized by using INS-1 cells in this present study, it showed that MK-4 is a potent stimulator of GSIS in INS-1 cells. GLP-1 is established to

amplify glucose-stimulated insulin secretion via the modulation of cAMP levels, it is well known that cAMP/PKA signaling pathway is involved in amplification of GSIS. However, Holz found that PKA is not the only protein that can bind to cAMP, which Epac (also known as cAMPGEFs, designated as cAMP-regulated guanine nucleotide exchange factors) are cAMP-dependent factors that promote insulin secretion (36). Here revealed that MK-4 stimulated cAMP levels but did not enhance PKA activity, it showed that MK-4 may regulate other factor participate in the signal transduction. Further studies about how MK-4 stimulates the signaling pathway in different cells should be conducted to clarify the mechanism of MK-4 in the near future.

To combine these issues, recent studies revealed that testosterone levels may modulate glycemic status in men, which mentioned that diabetes patients have significantly lower serum testosterone levels in comparison with men without diabetes, they also found that men with higher testosterone levels had a lower risk of type 2 diabetes (37, 38). The present study suggested that MK-4 and GGOH may raise testosterone levels in men, and MK-4 even exert antidiabetogenic effect. These findings provide novel mechanistic insight into the process of steroidogenesis and GSIS, and may be useful for the development of therapeutic strategies to men.

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