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碩士論文

冬蟲夏草刺激小鼠萊氏腫瘤細胞固醇類生成機制之探討

The Mechanism of *Cordyceps sinensis* on stimulating steroidogenesis in MA-10 Mouse Leydig Tumor Cells

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS i

INDEX TO FIGURES v

Abstract

Chinese viii

English x

INTRODUCTION 1

MATERIALS AND METHODS 9

Chemicals 9

Cell Culture and Experiments 10

Radioimmunoassay (RIA) 11

Protein Assay 12

Immunoblot Analysis 13

Statistics 13

RESULTS

Effect of CS on progesterone production in MA-10 cells 14

Effects of CS on cholera toxin-treated progesterone production in MA-10 cells 14

Effects of CS on GDP-β-S-treated progesterone production in MA-10 cells 15

Effects of CS on pertussis toxin-treated progesterone production in MA-10 cells 16

Effects of CS on forskolin-induced progesterone production in MA-10 cells 17

Effects of SQ 22536 on CS-stimulated progesterone production
in MA-10 cells ……..17
Effects of 2’ ,5’ dideoxyadenosine on CS-stimulated
progesterone production in MA-10 cells ……..…..18
Effects of CS on dbcAMP-induced progesterone production
in MA-10 cells …… …..… 18
Effects of Rp-cAMP on CS-stimulated progesterone production
in MA-10 cells ……..19
Effects of H89 and KT 5720 on CS-stimulated
progesterone production in MA-10 cells ……19
Effects of PKI on CS-stimulated progesterone production
in MA-10 cells …… …..… 20
Effect of actinomycin D on CS-stimulated steroidogenesis
in MA-10 cells …… …..… 20
Effect of cycloheximide on CS-stimulated steroidogenesis
in MA-10 cells …….. ……..20
Effect of mCCCP on CS-stimulated steroidogenesis
in MA-10 cells …….. ……..21
Effects of CS on StAR protein expression in MA-10 cells. ……..21
Effect of CS on the PMA-treated steroidogenesis in MA-10 cells ……..21
Effect of CS on the OAG-treated steroidogenesis in MA-10 cells ……..22
Effects of staurosporine on CS-stimulated steroidogenesis
in MA-10 cells …….. ……..22
Effect of calphostin C on CS-stimulated steroidogenesis
in MA-10 cells …….. ……..23

Effect of neomycin sulfate on CS-stimulated steroidogenesis
in MA-10 cells …… ……23
Effect of CS on the A23187-treated steroidogenesis
in MA-10 cells …… ……24
Effect of W7 on CS-stimulated steroidogenesis in MA-10 cells ……24
Effect of cadmium on CS-stimulated steroidogenesis
in MA-10 cells …… ……25
Effect of nifedipine on CS-stimulated steroidogenesis
in MA-10 cells …… ……25
Effect of flunarizine, dihydrochloride on CS-stimulated
steroidogenesis in MA-10 cells ……26
Effect of cotreatment of PKI and calphostin C on CS-stimulated
steroidogenesis in MA-10 cells ……26
Effect of CS on the 22R-hydroxycholesterol-treated
steroidogenesis in MA-10 cells ……27
Effect of P450scc enzyme inhibitor, aminoglutethimide
on CS-stimulated steroidogenesis in MA-10 cells ……27
Effect of CS on the pregnenolone-treated steroidogenesis
in MA-10 cells …… …… ……28
Effects of 3β-HSD enzyme inhibitor, trilostane on CS-stimulated
steroidogenesis in MA-10 cells ……28
DISSCUSSION …… …… ……60
REFERENCES …… …… . …… ……67
RESUME …… …… . …… …… ……82

Figure **Page**

The effect of CS on progesterone production in MA-10 cells ……29
The effects of CS on cholera toxin-treated steroidogenesis
in MA-10 cells ……30
The effect of GDP-β-S on CS-stimulated steroidogenesis
in MA-10 cells ……31
The effects of pertussis toxin (PTX) on CS-stimulated
steroidogenesis in MA-10 cells ……32
The effects of CS on forskolin-induced steroidogenesis
in MA-10 cells ……33
The effects of SQ 22536 on CS-stimulated steroidogenesis
in MA-10 cells ……34
The effects of 2’,5’ dideoxyadenosine on CS-stimulated
steroidogenesis in MA-10 cells ……35
The effects of CS on dbcAMP-induced steroidogenesis
in MA-10 cells ……36
The effect of Rp-cAMPS on CS-stimulated steroidogenesis
in MA-10 cells ……37
The effect of H89 on CS-stimulated steroidogenesis
in MA-10 cells ……38
The effect of KT 5720 on CS-stimulated steroidogenesis
in MA-10 cells ……39
The effect of PKI on CS-stimulated steroidogenesis
in MA-10 cells ……40
The effect of actinomycin D on CS-stimulated steroidogenesis
in MA-10 cells ……41

The effect of cycloheximide on CS-stimulated steroidogenesis
in MA-10 cells ……42
The effect of mCCCP on CS-stimulated steroidogenesis
in MA-10 cells ……43
The effects of CS on StAR protein expression in MA-10
cells ……44
The effect of CS on PMA-treated progesterone
production in MA-10 cells ……45
The effects of CS on 1-Oleoyl-2-acetyl-sn-glycerol- treated
progesterone production in MA-10 cells ……46
The effects of staurosporine on CS-stimulated
steroidogenesis in MA-10 cells ……47
The effect of calphostin C on CS-stimulated
steroidogenesis in MA-10 cells ……48
The effects of neomycin sulfate on CS-stimulated
steroidogenesis in MA-10 cells ……49
The effects of CS on A23187-treated steroidogenesis
in MA-10 cells ……50
The effect of W7 on CS-stimulated steroidogenesis
in MA-10 cells ……51
The effects of cadmium on CS-stimulated steroidogenesis
in MA-10 cells ……52
The effects of nifedipine on CS-stimulated steroidogenesis
in MA-10 cells ……53
The effects of flunarizine dihydrochloride on CS-stimulated
steroidogenesis in MA-10 cells ……54
The effect of cotreatment of PKI and calphostin C on CS-stimulated
steroidogenesis in MA-10 cells ……55

The effect of CS on 22R-hydroxycholesterol- treated
steroidogenesis in MA-10 cells ……..….56
The effect of aminoglutethimide on CS-stimulated
steroidogenesis in MA-10 cells ……..….57
The effect of CS on pregnenolone-treated
steroidogenesis in MA-10 cells …….. …..…..…..…..…..58
The effect of trilostane on CS-stimulated
steroidogenesis in MA-10 cells ……..….59

中文摘要

在先前的研究中我們發現，冬蟲夏草會刺激小鼠萊氏腫瘤 MA-10 細胞株產生黃體酮，目前為止相關的訊息傳導路徑仍所知有限。本論文將以冬蟲夏草處理 MA-10 細胞，並同時加入各種不同訊息傳導分子的活化劑或抑制劑，以確定冬蟲夏草刺激 MA-10 細胞的機制。首先，我們先檢測環腺嘌呤-蛋白激脢這條訊息傳遞路徑。實驗結果顯示，在冬蟲夏草刺激 MA-10 細胞產生黃體酮過程中可能會有至少一種 G 蛋白的參與；然而參與其中的 G 蛋白極有可能是 $G_{s\alpha}$而非 $G_{i\alpha}$的次單位。至於腺甘酸環化脢及蛋白激脢 A 的部分，實驗結果顯示冬蟲夏草可能不會影響腺甘酸環化脢活性，但卻透過活化蛋白質激脢 A 的訊號傳導路徑以促進 MA-10 細胞產生黃體酮。此外，蛋白質的新合成對冬蟲夏草刺激 MA-10 細胞產生黃體酮的效用也是必要的，而且西方墨點法的實驗結果也顯示，在以冬蟲夏草處理過的 MA-10 細胞其類固醇速控蛋白 (StAR protein)會增加表現，而且在冬蟲夏草刺激 MA-10 細胞使其產生黃體酮過程中，粒腺體內外膜氫離子濃度梯度也是必須的。由上可知，冬蟲夏草會藉由蛋白激脢 A 路徑來刺激 MA-10 細胞固醇類生合成，然而其效果僅佔約百分之三十七，這暗示我們會有其他的訊號傳遞路徑也可能參與其中。實驗結果顯示，蛋白激脢 C 路徑約佔冬蟲夏草刺激 MA-10 細胞固醇類生合成的百分

之五十二，而且磷脂脢 C 也參與在冬蟲夏草所刺激之效用中。令人驚訝的是，同時處理蛋白質激脢 A 及蛋白質激脢 C 抑制劑，冬蟲夏草刺激 MA-10 細胞的黃體酮生成會被抑制達百分之六十一；由此我們推論，冬蟲夏草刺激 MA-10 細胞的固醇類生合成會活化蛋白質激脢 A 及蛋白質激脢 C 之路徑。另一方面，鈣離子是另一個高度調控固醇類生合成的相關物質。而我們的結果顯示，鈣離子確實參與在冬蟲夏草刺激 MA-10 細胞固醇類生合成作用中，而且可能是透過 T 型而非 L 型鈣離子通道來作用。在固醇類生合成的酵素方面，冬蟲夏草會抑制細胞色素邊鏈切割酵素活性而不改變 3 beta–羥基類固醇脫氫脢的活性，使其達到影響 MA-10 細胞固醇類生合成的效果。至於冬蟲夏草在刺激固醇類生合成作用中卻抑制細胞色素邊鏈切割酵素的活性，必須再被探討。總結來說，冬蟲夏草刺激 MA-10 細胞固醇類生合成作用中，蛋白質激脢 A 訊息傳導路徑、蛋白質激脢 C 訊息傳導路徑、鈣離子以及固醇類生合成中其速率決定步驟的類固醇速控蛋白都扮演著重要的角色。

ABSTRACT

The stimulatory effects of crude CS on steroidogenesis in MA-10 mouse Leydig tumor cells have been demonstrated. However, the related signal transduction pathways are still unknown. MA-10 cells were treated with different reagents without or with CS (10 mg/ml) to determine the mechanisms. The cAMP-protein kinase A (PKA) signaling pathway was first examined. Results demonstrated that CS might activate at least one kind of G protein to affect production of progesterone in MA-10 cells, and $G_{s\alpha}$ subunit, but not $G_{i\alpha}$, might be involved in CS-stimulated steroidogenesis. With regards to adenylate cyclase and PKA, results indicated that adenylate cyclase might not be involved in CS-stimulated steroidogenesis, whereas PKA was activated by CS to stimulate MA-10 cell steroidogenesis. Moreover, de novo protein synthesis was required for CS-stimulated steroidogenesis. Besides, Western blot analysis illustrated that the expression of steroidogenic acute regulatory (StAR) protein increased in CS-treated cells. Result showed that the existence of mitochondria chemical gradient was also required for CS-stimulated steroidogenesis. PKA pathway involved almost 37% in CS-stimulated MA-10 cell steroidogenesis, which indicated that other pathways might also be involved. Thus, protein kinase C (PKC) pathway was examined. Results showed that activation of PKC was responsible for CS-stimulated steroidogenesis at about 52%, and phospholipase C was also required for CS-stimulated steroidogenesis. Therefore, both PKA and PKC signaling pathways were partially activated by CS in MA-10 cells, respectively.

Interestingly, the stimulatory effect of CS was inhibited 61% with cotreatment of PKA and PKC inhibitors, which confirmed that the effect of CS might simultaneously activate PKA and PKC pathways to stimulate MA-10 cell steroidogenesis. The role of calcium, which profoundly influences steroidogenesis, was also investigated. Results indicated that calcium might be involved in the stimulatory effect of CS on MA-10 steroidogenesis, and T-type calcium channel, but not L-typed, was required for the event. In steroidogenic enzyme activities, CS might inhibit P450 side chain cleavage enzyme activity, but not 3 beta-hydroxysteroid dehydrogenase enzyme, to regulate MA-10 cell steroidogenesis. In conclusion, CS might activate both PKA and PKC signal transduction pathways with calcium ion involved to stimulate steroidogenesis in MA-10 cells, and StAR protein played an important role in this stimulation.

INTRODUCTION

Cordyceps sinensis (Berk.) Sacc. is a fungus parasite on the larvae of Lepidoptera, which is also named as Dong-Chong-Xia-Cao (**冬蟲夏草**). It was initially recorded in **本草備要** by Wang-ang (**汪昂**) in 1694 and corrected by Wu-yiluo (**吳儀洛**) in **本草從新** during the Qing Dynasty in 1757. Cordyceps sinensis (CS) is a unique black, blade-shaped fungus found primarily at high altitude (3500-5000 meters) on the Qinghai-Tibetan plateau (**中國本草圖錄卷六**, 1989). In late autumn, the fungus attacks the caterpillars and is leisurely devouring its host. By early summer of the following year, the fungal infestation has killed the caterpillar and the fruiting body can be seen protruding from the caterpillar's head. Because of its particular life cycle, it is so called winter worm, summer-plant. Nevertheless, the supply of CS is inadequate to meet demand, particularly because it exists in high-altitude areas and is not easily harvested. However, the mycelium of the fungus has recently been cultured and a dried, powdered form of the mycelium is now commercially available.

The reason why CS is so popular in Chinese society might stem from its traditionally known pharmacological functions, such as the remedies on hypertension, hyperglycemia, hyperlipidemia, respiratory disease, renal dysfunction, and sexual dysfunction etc (Zhu et al, 1998a, b). According to records in traditionally Chinese medicine, CS goes to the lung merdian and kidney merdian, providing "lung protection," "kidney

improvement," and "Yin-Yang double invigoration." The kidneys are known as the root of life in traditional Chinese medicine, for that they store "Jing", the source of reproduction, development, and maturation (Kaptchuk, 1983). In fact, a number of bioactive constituents from CS have been reported, which include cordycepin (3'-deoxyadenosine) (Cunningham et al, 1951), modified nucleosides (Li et al, 2001), polysaccharides (Kiho et al, 1999), and sterols (Bok et al, 1999) etc.

Recent studies have illustrated that CS can potentiate the immune system (Yamaguchi et al, 1990; Yang et al, 1999), modulate TH1 and TH2 cell functions in bronchial airway (Kuo et al, 2001), and have antitumor activity (Yoshida et al, 1989; Bok et al, 1999; Nakamura et al, 1999). It can also be used to treat a broad range of disease, including decrease of blood pressure (Chiou et al, 2000), decrease of microorganism proliferation (White and Dawson, 1979), expansion of bronchial smooth muscles (Zhu et al, 1998b), increase of hepatic blood flow (Manabe et al, 2000), enhancement of hypoglycemic activity (Kiho et al, 1999), and implication for treatment of IgA mesangial nephropathy (Lin et al, 1999). Recent studies have also shown that CS stimulates corticosteroid production in cultured rat adrenocortical cells (Wang et al, 1998). Moreover, it has also been demonstrated that CS can enhance steroidogenesis in both normal mouse Leydig cells and MA-10 Leydig tumor cells (Huang et al, 2000; 2001a, b).

In male reproductive endocrine system, gonadotropin-releasing hormone (GnRH) from hypothalamus stimulates anterior lobe of pituitary

to release luteinizing hormone (LH). This trophic hormone will be transported to the testes to bind Leydig cells. After LH binds to its receptors, adenylate cyclase (AC) activities will be turned on by the heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) (Payne et al, 1996). This activation results in an increase in intracellular 3': 5'-cyclic adenosine monophosphate (cAMP), which in turn induces further activation of two separate, but equally important, pathways in support of the acute production of steroids. One of the pathways consists of the activation by phosphorylation of the enzyme cholesterol ester hydrolase, which converts cholesterol esters into free cholesterol (Stocco et al, 1996; 2000a). Free cholesterol is thought to be a precursor of all steroids, and movement of it from the outer to the inner mitochondrial membrane is the rate-limiting step in steroid hormone synthesis (Stocco et al, 1996; Christenson and Strauss, 2001). The biosynthesis of all hormonal steroids in all steroidogenic tissues begin with the enzymatic cleavage on cholesterol by the side chain cleavage enzyme (P450scc), which is located on the matrix side of the inner mitochondrial membrane (Simpson, 1979a). Once pregnenolone is formed, it may be metabolized within the mitochondria to progesterone by a mitochondrial 3β-hydroxysteroid dehydrogenase (3β-HSD), or it may exit the mitochondria and undergo further metabolism by microsomal cytochrome P450 steroid hydroxylases and steroid dehydrogenases with the final steroid hormone product dependent upon the nature of the tissue (Sulimovici et al, 1973; Cherradi et al, 1994). In addition to cholesterol mobilization to the outer membrane, the de novo synthesis of the steroidogenic acute regulatory (StAR) protein is also required to obtain

full steroidogenic capacity in response to hormone stimulation (Stocco, 2001). StAR is a 30 kDa phosphoprotein (Pon et al, 1986), which mediates the transfer of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450 side chain cleavage enzyme (P450scc) resides. Delivery of cholesterol to this enzyme constitutes the rate-limiting step in steroidogenesis (Cherradi et al, 1997). This step is rate limiting because the hydrophobic cholesterol can't transverse the aqueous intermembrane space of the mitochondria to reach the P450scc enzyme rapidly enough by simple diffusion to support acute synthesis (Christenson and Strauss, 2001). It has long been known that the biosynthesis of steroids is controlled by a cycloheximide sensitive factor whose function is to transfer cholesterol to the inner mitochondrial membrane (Simpson et al, 1979b). The StAR protein is an indispensable component in this process and is the best candidate to fill the role of the putative regulator (Stooco, 1996). Although it is clear that StAR plays a key role in regulating steroid hormone synthesis, the exact mechanism of StAR's participation in this process remains unknown. It was initially suggested that StAR must be imported into mitochondria to promote steroidogenesis, and that during the import process contact sites were formed between the outer and inner mitochondrial membranes, allowing cholesterol to flow down through a chemical gradient to reach P450scc. (Stocco and Clark, 1996; Cherradi et al, 1997). It has been demonstrated that N-terminal truncations of the StAR protein, removed as many as 62 amino acids, had no inhibitory effect on steroid production in transfected COS-1 cells (Arakane et al, 1996). These observations were soon confirmed by Wang et al (1998). Similar observations were demonstrated

that recombinant wild-type N-62 StAR protein enhanced pregnenolone production and the conversion of ^{3}H-cholesterol to ^{3}H-pregnenolone by isolated mitochondria (Arakane et al, 1998). Some experiments indicated that StAR could function as a sterol transfer protein. StAR was originally identified as a phosphoprotein, and initial experiments demonstrated a correlation between phosphorylated forms of StAR and steroidogenesis (Stocco and Sodeman, 1991). In addition, several models related to the transportation of cholesterol by StAR protein have been proposed based on a combination of structure/function studies or on the crystal structure of a related StAR-related lipid transfer (START) domain (Stocco, 2000b; Tsujishita and Hurley, 2000). No current models of StAR's mechanism of action as previously discussed can explain all known biochemical or biophysical properties attributed to this protein. Nevertheless, the control on the rate-limiting step by StAR protein in steroidogenesis remains to be examined.

Transduction of signals by a number of hormones are known to occur via the activation of AC, which increases intracellular cAMP levels and then activates protein kinase A (PKA) (De´saubry et al, 1996; Cooke, 1999). The criteria for cAMP being the second messenger for LH are satisfied in many aspects. LH increases cAMP in Leydig cells and its responses are potentiated at sub-maximal levels of LH by phosphodiesterase inhibitors (Moyle and Ramachandran, 1973). In addition, cAMP analogues (dibutyryl- and 8-bromo-cAMP) can mimic all stimulatory actions of LH (Cooke et al, 1977). Evidence has also been showed that activation of cAMP-dependent PKA can be detected with no

measurable change in the levels of cAMP (Moyle and Ramachandran, 1973). In MA-10 mouse Leydig tumor cells, it was shown that a cAMP derivative (Rp; cAMP phosphothioate), which acts as an antagonist of the activation of PKA, was a potent inhibitor on the steroidogenesis with the treatments of human chorionic gonadotropin (hCG), cholera toxin or 8-bromo-cAMP (Pereira et al, 1987).

The same stimulatory pathways may lead to desensitization and down-regulation of the LH receptor and steroidogenesis. Activation of PKC using the tumor-promoting phobol ester, phorbol-12-miristate-13-acetate (PMA), has been reported to regulate, both positively and negatively, steroidogenesis in Leydig cells (Cooke et al, 1992). Under a saturating concentration of LH, which stimulates maximally cAMP and testosterone syntheses, exogenous phospholipase C (PLC) and PMA inhibit this process in dose- and time-dependent manners (Papadopoulos et al, 1985). But, the results are different from those of Mukhopadhyay et al. (1984), who found that PMA has no effect on testosterone synthesis in mouse Leydig cell suspensions in the presence of a saturating concentration of LH. Indeed, when sub-maximal dosage of LH is used, PMA does inhibit testosterone synthesis. LH/hCG doesn't stimulate inositol phosphate accumulation in MA-10 cells (Ascoli et al, 1989). However, a recent study suggests that the undetectable inositol phosphate in hCG-induced steroidogenesis in MA-10 cells was due to their low density of endogenous LH receptors (Hirakawa et al, 2002). The LH receptor exists in a dynamic state being truncated, internalized, degraded or recycled (Cooke et al, 1992). Desensitization is controlled by PKC in

the rat and by PKA and PKC in the mouse Leydig cells (Inoue and Rebois, 1989).

Most of the available evidences indicate that, in addition to cAMP, calcium (Ca^{2+}) is likely to be involved in the activation of steroidogenesis by LH/hCG (Sullivan and Cooke, 1986). Thus, like its effects on the gonadotrophs cells (Conn et al, 1986), the activation of steroidogenesis in rat Leydig cells by GnRH is believed to be mediated by Ca^{2+} (Cooke and Sullivan, 1985). Other findings indicating an involvement of Ca^{2+} in the activation of gonadal steroidogenesis include many instances such as (1) removal of extracellular Ca^{2+} (Janszen et al, 1976; Manna et al, 1999), addition calmodulin antagonist (Hall et al, 1981) or Ca^{2+} channel blocker (Manna et al, 1999) has been shown to blunt the steroidogenic response to maximally effective concentrations of LH/hCG and/or cAMP analogues in Leydig cells; (2) addition liposomes containing Ca^{2+} and calmodulin to rat Leydig cells has also been reported to enhance steroid biosynthesis and the transport of cholesterol to mitochondria (Hall et al, 1981); and (3) ionophretic mobilization of Ca^{2+} has been shown to induce a modest increase in steroid biosynthesis in Leydig cells (Sullivan and Cooke, 1984; Lin, 1985).

It has been demonstrated that CS can increase MA-10 cell steroid synthesis (Huang et al, 2000; 2001a). However, it is still unclear how CS influences the signal transduction pathways to stimulate Leydig cell steroidogenesis. In the present study, we would like to evaluate the mechanism of CS in MA-10 cell steroidogenesis. Thus, various reagents

without or with CS (10 mg/ml) were applied to cells for 3 hr, and progesterone levels were analyzed by radioimmunoassay (RIA). Moreover, the expression of StAR protein was examined, to determine the mechanism how CS regulate MA-10 cell steroidogenesis.

MATERIALS & METHODS

Chemicals

The Simpson Biotech Co. Ltd. constantly supplied culture mycelium of Cordyceps sinensis. A polyclonal antibody generated against residues 89-99 of human StAR was a gift from Dr. Jerome Struass, III (University of Pennsylvania, PA, USA). Trilostane was a gift from Dr. Wendy Chang (Sanofi-Synthelabo Research, Malvern, PA). Fetal bovine serum, and lyophilized trypsin-EDTA were purchased from Gibco (Grand Island, NY). Waymouth MB 752/1 medium, bovine serum albumin (BSA), forskolin, cholera toxin, pertussis toxin, guanosine 5' -[β-thio]diphosphate trilithium salt (GDP-β-S), staurosporine, dextran T-70, dimethyl sulphoxide (DMSO), sodium azide, DL-aminoglutethimide, cycloheximide, K^+-Na^+-tartrate, N-(2-[p-Bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H89), human chorionic gonadotropin (hCG) (10,000 IU per gram), dibutyryl-adenosine 3' : 5' -cyclic monophosphate, 22R-hydroxycholesterol, pregnenolone, phorbol-12-miristate-13-acetate (PMA), copper sulfate, nifedipine, carbonyl cyanide m-chlorophenylhydrazone (mCCCP) and W7 were marketed by Sigma Chemical Co. (St. Louis, MO). Tissue culture grade sodium bicarbonate, sodium carbonate, sodium chloride, sodium dihydrogen phosphate, potassium chloride and cadmiumchlorid-1-hydrat were purchased from Riedel-deHaen (Seelze, Germany). Sodium hydroxide and hydrochloric acid were purchased from Merck (Darmstadt, Germany). EDTA was bought from Bio-Rad Laboratories (Richmond,

CA). Sucrose, Tris (base) and sodium hydrogen phosphate were purchased from J.T. Baker (Phillipsburg, NJ). Charcoal was purchased from Showa Chemical Inc. (Tokyo, Japan). SQ 22536 was purchased from Sigma RBI (St. Louis, MO). Gelatin was obtained from Difco Laboratories (Detroit, MI). HEPES was purchased from Mallinckrodt Baker, Inc. (Paris, Kentucky). Gentamycin sulfate, folin-ciocalteu's phenol reagent and actinomycin D were purchased from Fluka Chemical Co. (Buchs, Switzerland) Calphostin C, PKI (14-22 amide), neomycin sulfate, 1-oleoyl-2-acetyl-sn-glycerol (OAG), flunarizine dihydrochloride, 2', 5' dideoxyadenosine and KT5720 were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Rp-cAMPS, triethylammonium salt and Melittin were purchased from Tocris Cookson Ltd. (Ellisville, MO). ^{3}H-progesterone used for radioimmunoassay was purchased from PerkinElmer Life Sciences Inc. (Boston, MA). Antiserum to progesterone was obtained from Holly Hills Biologicals Inc. (Hillsboro, OR) and Dr. Paulus Shyi-Gang Wang (University of Yang-Min, Taipei, Taiwan).

Cell Culture and Experiments

The MA-10 cell line was a gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA) and was maintained according to standard procedures (Ascoli, 1981). This MA-10 cell line is widely used as a model system for investigations on the regulation of steroidogenesis. The cells can be grown in culture and possess functional LH/hCG receptors that mediate a very strong stimulation of progesterone production (Ascoli,

1981). The cells show all the features of normal Leydig cells, except that the amount of P450c17 mRNA and P450c17 activity is insignificant (Mellon SH, 1989). Consequently, the production of androgens by these Leydig cells is almost undetectable; and instead, large quantities of progesterone are produced (Ascoli, 1981; Mellon, 1989) and respond to both trophic hormones (LH and hCG) and cAMP analogues (Ascoli, 1981; Huang et al, 1995). Cells (5×10^4) were plated in 96-well plates and grown for 24 hr in Waymouth 752/1 MB medium containing 13 mM $NaHCO_3$, 24 mM HEPES, 0.04 g gentamycin sulfate, and 15% fetal bovine serum, pH 7.7. At this time, the medium was removed and the cells were washed twice with PBS buffer. The effects of CS on steroidogenesis were subsequently determined. Cells were maintained in a humidified atmosphere containing 95% air, 5% CO_2 at 37^oC incubation. After each experiment, media were collected and stored at -20^oC until assayed for progesterone by RIA.

Radioimmunoassay (RIA)

The antiserum to progesterone was used at the dilution of 1:10,000 in 0.1% PBS gel. Progesterone was obtained from sigma Chemical Co. and used for standards. A stock solution (1 pg/ml) was prepared in absolute ethanol and concentrations of 5, 10, 20, 35, 50, 75, 100, 150, 200, 300, and 500 pg/µl were prepared. Media from cultures with different reagent treatments were collected and diluted with medium to fall in the standard curves for the respective assays. Twenty-five µl of diluted sample was withdrawn to individual 12x75 mm assay tubes and 100 µl of

progesterone antiserum and 100 μl of ^{3}H-progesterone (specific activity =90-115 Ci/mmol) were added. After mixing, the tubes were incubated at 37 ^{o}C for 30 min. The tubes were then placed in an ice-bath and added 0.5% PBS gel to stop the reaction. Charcoal was added and incubated for 15 min at 4°C and then centrifuged for 10 min to spin down the charcoal bound with free ^{3}H-steroids. The supernatant was poured into 3 ml of scintillation fluid and samples were counted in a β-counter (Beckman Coulter Instruments Inc., Fullerton, CA) for 2 min per sample. The sensitivity of the assay was 5 pg per assay tube. The progesterone level in each treatment was then normalized by the total protein concentration measured by Lowry protein assay method (Lowry et al, 1951).

Protein Assay

After the experiment in which progesterone was measured, cells in each well were solubilized in one hundred μl of 0.5N hydrochloric acid (HCl). Fifty μl cell lysate was poured into 50 μl Tris Sucrose EDTA (TSE) buffer consisting of 10 mM Tris (base), 0.25 mM sucrose, 0.1 mM EDTA, pH 7.4. 1 ml reagent I were added to cells and the reagent I was containing 2% sodium bicarbonate, 0.5% cupper sulfate, 1% K^{+}-Na^{+}-tartrate, and 1N sodium hydroxide for 10 min. The proportional of reagent I was 49: 1: 1: 2. After 10 min, 1N folin were added to cells for 30 min. Protein concentrations were measured at 750 nm with a spectrophotometer (DU 640; Beckman Coulter Instruments Inc., Fullerton, CA)

Immunoblot Analysis

The total protein was solubilized in a sample buffer consisting of 25 mM Tris/HCl, pH 6.8, 1% SDS, 5% mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue and loaded on 12.5% SDS-PAGE minigels (Mini-Protein II System; Bio-Rad, Richmond, CA). Electrophoresis was performed at 200 V for 40 min using standard SDS-PAGE running buffer (24 mM Tris/HCl, 0.19 M glycine, 0.5% SDS, pH 8.3), and the proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad) at 100 V for 2 hr in transfer buffer (20 mM Tris/HCl, 150 mM glycine, 10% methanol, 0.01% SDS). Immunodetection of the 30-kDa StAR protein using rabbit polyclonal antisera to the StAR protein was performed as previously described (Ju et al, 2000, Wu et al. 2001). The appropriate protein bands of interest were quantitated using a Quantity One (PDI, Huntington Station, New York) computer-assisted image analysis system. The data for protein quantitation was expressed as integrated optical density, which had been normalized.

Statistics

Each data point in the figures represents the mean±SEM in percentage of progesterone production in three experiments with triplicates in each treatment or StAR protein integrated optical density in percentage of three experiments. Statistically significant differences between treatments and control were determined by one-way analysis of variance (ANOVA) and the least significance difference (LSD).

RESULTS

Effect of CS on progesterone production in MA-10 cells

The MA-10 cells were treated with vehicle, hCG (50 ng/ml), CS (10 mg/ml) and CS plus hCG (Fig. 1). Progesterone production in hCG or CS treatment alone was significantly increased as compared to the vehicle ($p<0.05$). In addition, the progesterone level in the treatment of CS with hCG was much higher than CS alone treatment ($p<0.05$). These four principle treatments were used to evaluate the condition of MA-10 cells. If progesterone production in hCG and CS treatments were not different from that of control group, whole experiment data would not be included for analysis.

Effects of CS on cholera toxin-treated progesterone production in MA-10 cells

Steroidogenesis in Leydig cell is regulated by LH/hCG through specific cell surface receptors to activate intracellular effector systems, and thus to increase cholesterol metabolism. A number of studies have suggested that the cAMP-PKA system is the major signal transduction pathway involved in the regulation of Leydig cell steroid synthesis (Cooke, 1999; Ascoli et al, 2002). Thus, we examined if CS would affect any locus along cAMP-PKA pathway. The LH/hCG receptor is a G protein coupled receptor. In order to know if CS influenced the $G_{s\alpha}$ protein to stimulate MA-10 cell steroidogenesis, different concentrations of $G_{s\alpha}$ protein activator, cholera toxin (CTX) (1-100 ng/ml), without or

with CS (10 mg/ml), were added to cells for 3 hr incubation. The α subunit of CTX is an enzyme that penetrates the plasma membrane and enters the cytosol, where it catalyzes the covalent addition of the ADP-ribose moiety from intracellular NAD^+ to $G_{s\alpha}$. ADP-ribosylated $G_{s\alpha}$ GTP can activate AC normally but can't hydrolyze the bound GTP to GDP; thus $G_{s\alpha}$ remains in active state to continuously activate AC (Gudermann et al, 1996). As shown in figure 2, CTX (1-100 ng/ml) caused a dosage-dependent increase in progesterone production. CTX at 100 ng/ml significantly stimulated progesterone production, as compared to the control group. Progesterone production in MA-10 cells following CTX (10 and 100 ng/ml) treatment was significantly enhanced in the presence of CS, as compared to CS alone treatment ($p<0.05$). These results indicated that CS might affect $G_{s\alpha}$ protein to stimulate MA-10 cell steroidogenesis.

Effect of GDP-β-S on CS-stimulated progesterone production in MA-10 cells

We further examined if the inhibition of G protein activation could abolish the stimulation of steroidogenesis. The general G protein antagonist, GDP-β-S, which is known as a GDP analogue, competes with intracellular GTP to inhibit G protein activation (Gilman, 1987; Piacentini et al, 1996). Cells were treated without or with GDP-β-S (100 μM) plus CS (10 mg/ml) for 3 hr incubation. As shown in figure 3, GDP-β-S (100 μM) alone didn't induce progesterone production. However, GDP-β-S (100 μM) reduced CS-stimulated steroidogenesis by 18 ± 2% ($p<0.05$). It suggested that CS-stimulated progesterone

production is mediated at least in part, by G protein.

Effects of pertussis toxin on CS-stimulated progesterone production in MA-10 cells

G proteins are heterotrimers composed of α, β, and γ subunits. They are classified based on amino acid sequence similarity of their α subunits and subdivided into four families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$. (Simon et al, 1991). G_i protein is inhibited by pertussis toxin catalyzing addition of ADP-ribose to the α subunit of G_i, which causes an irreversible releasing of GDP and inactivates G_i protein will sustain (Nikula and Huhtaniemi, 1989). In the present study, different concentrations of $G_{i\alpha}$ protein inhibitor, pertussis toxin (PTX) (10 ng/ml-1 μg/ml), without or with CS (10 mg/ml) were added to cells for 3 hr incubation. Figure 4 illustrated that there were no significant differences among various concentrations of PTX plus CS treatments compared to the CS alone treatment ($p>0.05$). In addition, PTX at different dosages didn't induce progesterone production compared to control ($p>0.05$). These data suggested that G_i protein might not be involved in CS-stimulated steroidogenesis.

Effects of CS on forskolin-induced progesterone production in MA-10 cells

Adenylate cyclases are a family of membrane-bound enzymes that catalyze the formation of cAMP from ATP, and are regulated by numerous neurotransmitters and hormones via G protein-linked cell surface receptors (Mathew and Vanholde, 1992). The activity of

adenylate cyclase can be regulated directly by forskolin and by certain adenosine derivatives via P site (De´saubry et al, 1996). To investigate the possible site where CS might act along the signal transduction pathway to affect MA-10 cell steroidogenesis, cells were treated with forskolin (1-100 μM) without or with CS (10 mg/ml) for 3 hr incubation. Figure 5 illustrated that forskolin caused a dosage-dependent increase in progesterone production. Forskolin at 100 μM significantly increased progesterone production compared to control group. The forskolin-induced progesterone production was significantly increased by CS when forskolin at 50 μM ($p<0.05$). These data suggested that CS might enhance cAMP-PKA pathway to affect MA-10 cell steroidogenesis.

Effects of SQ 22536 on CS-stimulated progesterone production in MA-10 cells

To confirm the effect of CS on adenylate cyclase enzyme, we used SQ 22536 (SQ) to block adenylate cyclase activity, which inhibits adenylate cyclases by blocking P-site of adenylate cyclase (Londos and Wolff, 1977; Tsai et al, 1997). Cells were treated without or with SQ (1-100 μM) plus CS (10 mg/ml) for 3 hr incubation. There were no significant differences among various concentrations of SQ (1-100 μM) plus CS treatments compared to the CS alone treatment ($p>0.05$). In addition, SQ at different dosages didn't induce progesterone production compared to basal ($p>0.05$). These data suggested that adenylate cyclase might not be involved in CS-stimulated steroidogenesis.

Effects of 2',5' dideoxyadenosine on CS-stimulated progesterone production in MA-10 cells

Another potent adenylate cyclase inhibitor, 2',5' dideoxyadenosine (2',5' -DDA), was also used in present study. It is also reported that 2',5' dideoxyadenosine is a P-site inhibitor of adenylate cyclase (Hurley, 1998; De´saubry et al, 1996). Different concentrations of 2',5' DDA (0.3-30 μM) couldn't inhibit CS-stimulated steroidogenesis (Fig. 7). There were no significant differences among various concentrations of 2',5' DDA plus CS treatments compared to the CS alone treatment ($p>0.05$). In addition, 2',5' DDA at different dosages didn't induce progesterone production compared to basal ($p>0.05$). These results (Fig. 5, 6, 7) again suggested that CS might not affect adenylate cyclase to stimulate steroidogenesis in MA-10 cells.

Effects of CS on dbcAMP-induced progesterone production in MA-10 cells

cAMP-dependent protein kinase (PKA) is a major pathway to stimulate Leydig cell steroid synthesis (Payne et al, 1996). Dibutyryl-cAMP is a cAMP analogue, which can activate PKA to stimulate steroidogenesis. dbcAMP (0.05-1.0 mM) caused a dosage-dependent increase in progesterone production (Fig. 8), and dbcAMP plus CS (10 mg/ml) increased much higher progesterone production. The dbcAMP-induced progesterone production was significantly increased by CS when the dosage of dbcAMP was at 0.25 mM ($p<0.05$). The results indicated that CS might affect PKA to stimulate steroidogenesis.

Effect of Rp-cAMPS on CS-stimulated progesterone production in MA-10 cells

In order to confirm the effect of CS on PKA, we used Rp-cAMPS to block PKA activity. Rp-cAMPS blocks signal transduction of cAMP by antagonizing the cAMP binding sites of protein kinase A (Pereira et al, 1987). Cells were pretreated with Rp-cAMPS (Rp) (200 μM) for 30 min. After that, cells were treated without or with Rp (200 μM) plus CS (10 mg/ml) for 3 hr incubation. The result showed that Rp (200 μM) didn't inhibit CS-stimulated progesterone production, which might due to its inhibitory concentration was not reached ($p>0.05$) (Fig. 9). Thus higher concentration might need to be reconfirmed.

Effects of H89 and KT 5720 on CS-stimulated progesterone production in MA-10 cells

H89 and KT 5720, PKA inhibitors can selectively inhibit PKA activation by blocking ATP binding site in PKA (Davies et al, 2000; Wang et al, 2001; Sirotkin and Makarevich, 1999). Thus, both reagents were used to examine if CS would activate PKA pathway. Cells were pretreated with H89 (50 μM) (Fig. 10) or KT 5720 (KT) (6 μM) (Fig. 11) for 30 min, respectively. After 30 min, cells were treated without or with H89/ KT plus CS (10 mg/ml) for 3 hr incubation. The result showed that H89 (50 μM), but not KT 5720 (6 μM), could inhibit CS-stimulated progesterone production. H89 (50 μM) significantly inhibited CS-stimulated progesterone production by 37 ± 9% ($p<0.05$). These results suggested that CS might activate PKA to stimulate MA-10 cell steroidogenesis.

Effect of PKI on CS-stimulated progesterone production in MA-10 cells

Protein kinase A inhibitor, PKI, selectively inhibits PKA activation by blocking catalytic subunit of PKA (Eichholtz, 1993). To reconfirm that CS might activate PKA, PKI was used in this study. Cells were treated without or with PKI (4 μM) plus CS (10 mg/ml) for 3 hr incubation. As shown in figure 12, PKI (4 μM) significantly reduced CS-stimulated steroidogenesis by 40 ± 6% ($p<0.05$). These results (Fig. 9, 10, 11, and 12) indicated that CS might activate PKA pathway to stimulate MA-10 cell steroidogenesis.

Effect of actinomycin D and cycloheximide on CS-stimulated steroidogenesis in MA-10 cells

It has been demonstrated that de novo protein synthesis is essential for steroid production in Leydig cells (Huang et al, 2001b; Stocco, 1996; Ramnath et al, 1997). It is also important to know if CS will induce new protein synthesis to stimulate steroidogenesis. Thus, cells were treated without or with transcriptional inhibitor, actinomycin D (Act-D) (10 μg/ml) or protein synthesis inhibitor, cycloheximide (CHX) (1 mM) plus CS (10 mg/ml) for 3 hr incubation. Figure 13 demonstrated that Act-D at 10 μg/ml inhibited CS-stimulated progesterone production by 71 ± 9% ($p<0.05$). The result indicated that transcriptional regulation might be involved in CS-stimulated MA-10 cell steroidogenesis.However, progesterone production stimulated by CS in MA-10 cells was significantly reduced (48 ± 6%) in the presence of CHX (Fig. 14) ($p<0.05$). The result indicated that new protein synthesis is necessary in CS-stimulated MA-10 cell steroidogenesis.

Effect of mCCCP on CS-stimulated steroidogenesis in MA-10 cells

Electrochemical gradient across the inner mitochondrial membrane is required for the StAR to import cholesterol for steroidogenesis (Huang et al, 1997b; King et al, 1999). It is possible that CS may also need this gradient to stimulate steroidogenesis. mCCCP, a well-characterized reagent that equilibrates the proton gradient across the inner mitochondria membrane, was used to determine this phenomenon (Tzagoloff, 1982). Cells were treated without or with protonophore, mCCCP (20 μM), plus CS (10 mg/ml) for 3 hr incubation. Progesterone production stimulated by CS in MA-10 cells was significantly reduced (43 ± 5%) in the presence of mCCCP (Fig. 15) ($p<0.05$). The result implied that electrochemical gradient of mitochondria might be involved in CS-stimulated MA-10 cell steroidogenesis.

Effects of CS on StAR protein expression in MA-10 cells

Since StAR protein plays an important role in steroidogenesis, we examined if CS would up-regulate the expression of StAR. Cells were treated with vehicle, hCG 50 ng/ml, CS 10 mg/ml, CS 10 mg/ml plus hCG, CS 2 mg/ml, CS 2 mg/ml plus hCG for 3 hr incubation. Figure 16 illustrated that CS 10 mg/ml could activate the expression of StAR. Moreover, CS 10 mg/ml plus hCG enhanced more progesterone production in MA-10 cells.

Effect of CS on the PMA-treated steroidogenesis in MA-10 cells

Since PKA inhibitors reduced 30-40% steroidogenesis in MA-10 cells, it is possible that other signal pathways may also be involved. In

steroidogenic cells, PKC also plays an important role to regulate steroidogenesis. Thus, PKC pathway was examined. Phorbol ester, PMA as PKC activator (2.5 μg/ml) (Rebois and Patel, 1985; Inoue and Rebois, 1989), was added to cells without or with CS (10 mg/ml) for 3 hr incubation. As shown in figure 17, PMA (2.5 μg/ml) significantly enhanced progesterone production by 262 ± 82% in the presence of CS. The result suggested that CS might activate PKC pathway to stimulate steroidogenesis.

Effects of CS on the OAG-treated steroidogenesis in MA-10 cells

Another PKC activator, OAG functioning as DAG analogue to activate PKC, was also used to determine if CS might activate PKC pathway (Nikula and Huhtaniemi, 1989; Nishizuka, 1992). Cells were treated without or with OAG (1-100 μM) plus CS (10 mg/ml) for 3 hr incubation. Figure 18 demonstrated that there were no significant differences between CS and OAG (1-100 μM) plus CS treatments. Base on the data, we couldn't exclude the role of diacylglycerol on CS-stimulated steroidogenesis.

Effects of staurosporine on CS-stimulated steroidogenesis in MA-10 cells

To confirm the role of PKC in CS-stimulated steroidogenesis, we used staurosporine (Ss), a non-specific PKC inhibitor, to block PKC activation. Ss inhibits PKC activation by blocking ATP binding site of PKC (Kumar et al, 1994; Lee and Stern, 2000). Cells were treated without or with Ss (0.1-10 nM) plus CS (10 mg/ml) for 3 hr incubation.

The stimulatory effect of CS couldn't be blocked by Ss (0.1-10 nM), which might due to the dosage was not enough to work, and further study might need to be conducted (Fig. 19) ($p>0.05$).

Effect of calphostin C on CS-stimulated steroidogenesis in MA-10 cells

Calphostin C, which is a specific PKC inhibitor by blocking the binding site of diacylglycerol of regulatory domain of PKC, was also used to determine the role of PKC in CS-stimulated steroidogenesis (Wang et al, 1998). Cells were treated without or with calphostin C (Cal) (500 nM) plus CS (10 mg/ml) for 3 hr incubation. Progesterone production stimulated by CS in MA-10 cells was significantly reduced (52 ± 18%) in the presence of Cal (Fig. 20) ($p<0.05$). These results (Fig. 17 and 20) indicated that activation of PKC was responsible for the CS-stimulated steroidogenesis in MA-10 cells.

Effect of neomycin sulfate on CS-stimulated steroidogenesis in MA-10 cells

In response to trophic factors or agonists in PKC signaling pathway, diacylglycerol is produced as a result of hydrolysis of inositol phospholipids, particularly phosphatidylinositol 4, 5-bisphosphate (PIP_2). However, hydrolysis of PIP_2 is mediated by phospholipase C (PLC) enzyme (Nishizuka, 1992). To determine the role of PLC in CS-stimulated steroidogenesis, PLC inhibitor, neomycin sulfate, was used in the study. Neomycin sulfate is reported as a PLC inhibitor by blocking PIP_2 hydroxylation (Somjen et al, 1997). Cells were treated without or with neomycin sulfate, (Neo) (5-500 μM) plus CS (10 mg/ml) for 3 hr

incubation. As shown in figure 21, Neo at 5, 50 and 500 μM reduced CS-stimulated steroidogenesis by 35 ± 9%, 43 ± 1% and 37 ± 9%, respectively ($p<0.05$). The result suggested that PLC enzyme activity was also important for CS-stimulated steroidogenesis in MA-10 cells.

Effects of CS on the A23187-treated steroidogenesis in MA-10 cells

Many evidences indicate that, in addition to cAMP, calcium also plays important roles in the control of gonadal steroid biosynthesis (Sullivan and Cooke, 1986). Thus, the role of calcium in CS-stimulated steroidogenesis was also determined. Cells were treated without or with calcium ionophore, A23187 (0.5-50 μM), plus CS (10 mg/ml) for 3 hr incubation (Pereira et al, 1988). As shown in figure 22, A23187 (0.5-50 μM) enhanced CS-stimulated steroidogenesis in a dosage dependent manner. Moreover, A23187 (50 μM) significantly increased more progesterone production in the presence of CS ($p<0.05$). In addition, A23187 alone at different dosages didn't induce progesterone production compared to basal ($p>0.05$). The results indicated that increased intracellular calcium concentration was required for CS-stimulated steroidogenesis.

Effect of W7 on CS-stimulated steroidogenesis in MA-10 cells

It has been reported that the addition of liposomes containing calcium and calmodulin to rat Leydig cells will enhance steroid synthesis (Hall et al, 1981). To investigate the role of intracellular calcium related to CS stimulatory effect, cells were treated without or with calmodulin antagonist, W7 (10 μM), plus CS (10 mg/ml) for 3 hr incubation. W7 is

calmodulin antagonist by blocking the binding site of calmodulin (Itoh and Hidaka, 1984). As shown in figure 23, W7 (10 μM) significantly reduced CS-stimulated steroidogenesis by 45 ± 3% ($p<0.05$). The result again suggested that CS affected MA-10 cell steroidogenesis with calcium involved.

Effects of cadmium on CS-stimulated steroidogenesis in MA-10 cells

It has been demonstrated that LH-stimulated testosterone production is inhibited in the absence of extracellular calcium in mouse Leydig cells (Manna et al, 1999). Cells were treated without or with non-selective calcium channel blocker, cadmium (0.01-1 mM), plus CS (10 mg/ml) for 3 hr incubation. Cadmium is a potent inhibitor of voltage-dependent calcium channel and its inhibition involves competition with calcium for the cation binding sites in the channel pores (Usai, C et al, 1999). Figure 24 showed that CS-stimulated steroidogenesis was significantly reduced by cadmium in a dosage dependent manner. Cadmium at 0.1 and 1 mM significantly reduced CS-stimulated progesterone levels by 88 ± 2% and 96 ± 4%, respectively ($p<0.05$). The results suggested that extracellular calcium influx was required for CS-stimulated steroidogenesis.

Effects of nifedipine on CS-stimulated steroidogenesis in MA-10 cells

In order to know which type of calcium channel was involved in CS-stimulated steroidogenesis. L-type calcium channel blocker, nifedipine (5-500 μM), was added to cells without or with CS (10 mg/ml) for 3 hr incubation. Nifedipine is reported as dihydropyrdine-sensitive (L-type) channel blocker, which binds to the dihydropyrdine binding sites

in the channel pores (Striessnig et al, 1998). As shown in figure 25, different concentrations of nifedipine (5-500 μM) didn't block CS-stimulated steroidogenesis ($p>0.05$). The result demonstrated that L-type calcium channel might not participate in CS-stimulated steroidogenesis

Effects of flunarizine dihydrochloride on CS-stimulated steroidogenesis in MA-10 cells

There are several types of voltage-gated channel, including N, L, P, Q, O, R, and T type calcium channels (Mathew and Van holde, 1992). T-type calcium channel blocker, flunarizine dihydrochloride (flu) (0.2-20 μM), was added to cells without or with CS (10 mg/ml) for 3 hr incubation. flu inhibits T-type calcium channel by antagonizing with calcium for the cation binding sites in the channel pores (Miller, 1987). As shown in figure 26, flu (20 μM) blocked CS-stimulated steroidogenesis ($p<0.05$). The result demonstrated that T-type calcium channel might be involved in CS-stimulated steroidogenesis.

Effect of cotreatment of PKI and calphostin C on CS-stimulated steroidogenesis in MA-10 cells

To examine if both PKA and PKC pathways were activated by CS in MA-10 cells, cells were treated without or with PKI (4 μM) and calphostin C (500 nM) plus CS (10 mg/ml) for 3 hr incubation. Figure 27 illustrated that PKI cotreated with calphostin C inhibited CS-stimulated progesterone production by 61 ± 8% ($p<0.05$). It suggested that the stimulatory effect of CS might partially go through protein kinase A

pathway and partially go through protein kinase C pathway, simultaneously.

Effect of CS on the 22R-hydroxycholesterol-treated steroidogenesis in MA-10 cells

To examine if CS might directly affect the activity of steroidogenic enzyme, the water-soluble cholesterol analogue, 22R-hydroxycholesterol (22R) (50 μM) was used to treat cells. 22R freely passes from cytoplasm to the inner mitochondrial membrane where it is converted to pregnenolone by P450scc enzyme (Gyles et al, 2001). MA-10 cells were treated without or with 22R (50 μM) plus CS (10 mg/ml) for 3 hr incubation. Figure 28 demonstrated that CS significantly reduced 22R-treated progesterone production ($p<0.05$). The result suggested that CS might inhibit P450scc enzyme to affect MA-10 cell steroidogenesis.

Effect of P450scc enzyme inhibitor, aminoglutethimide, on CS-stimulated steroidogenesis in MA-10 cells

In order to confirm the effect of CS on P450scc enzyme, we used P450scc inhibitor, aminoglutethimide (AG), to block the conversion of cholesterol to pregnenolone. AG appears to act by competing with cholesterol for the substrate binding site of P450scc enzyme (Ascoli et al, 1983). Cells were treated without or with AG (0.2-20 μM) plus CS (10 mg/ml) for 3 hr incubation. Figure 29 showed that AG (0.2-20 μM) significantly reduced CS-stimulated steroidogenesis by 60-88% in a dosage dependent manner ($p<0.05$). These results (Fig. 28 and 29)

demonstrated that CS might inhibit P450scc enzyme to influence MA-10 cell steroidogenesis.

Effect of CS on the pregnenolone-treated steroidogenesis in MA-10 cells

In steroidogenic pathway, cholesterol is converted to pregnenolone by P450scc enzyme. Pregnenolone is then converted to progesterone by 3β-HSD enzyme (Liu et al, 2001). To examine if CS acted on 3β-HSD enzyme, cells were treated without or with pregnenolone (preg) (50 μM) plus CS (10 mg/ml) for 3 hr incubation. Figure 30 showed that preg-treated progesterone production was not affected by CS ($p>0.05$). The result indicated that CS might not affect 3β-HSD enzyme for steroidogenesis.

Effects of 3b-HSD enzyme inhibitor, trilostane, on CS-stimulated steroidogenesis in MA-10 cells

In order to confirm if CS affected 3β-HSD enzyme for MA-10 cell progesterone production, the 3β-HSD enzyme inhibitor, trilostane (tri), was used (Lukyanenko et al, 1998). Cells were treated with CS without or with 3β-HSD enzyme inhibitor, tri (1-100 μM), for 3 hr incubation. Figure 31 showed that tri (1-100 μM) completely reduced CS-stimulated progesterone production near to the basal level ($p<0.05$). These results (Fig. 30 and 31) demonstrated that CS might not act on 3β-HSD enzyme to influence MA-10 cell steroidogenesis.

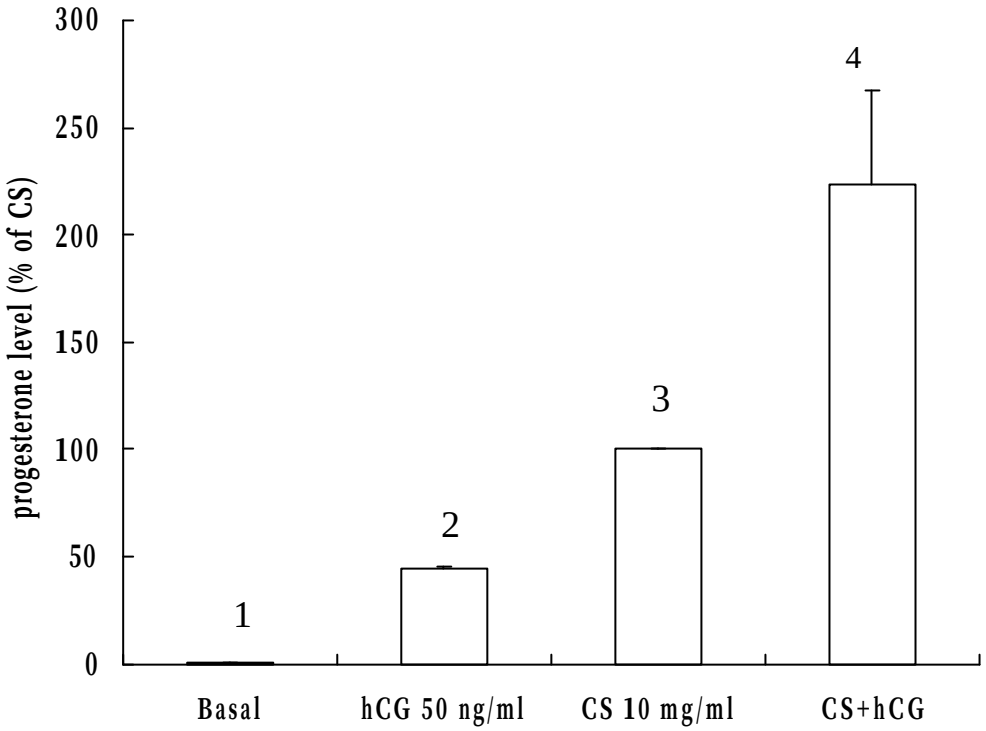

Figure 1. The effect of CS on progesterone production in MA-10 cells. Cells were treated without or with hCG (50 ng/ml) plus CS (10 mg/ml) for 3 hr incubation. CS enhanced hCG-stimulated steroidogenesis. Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

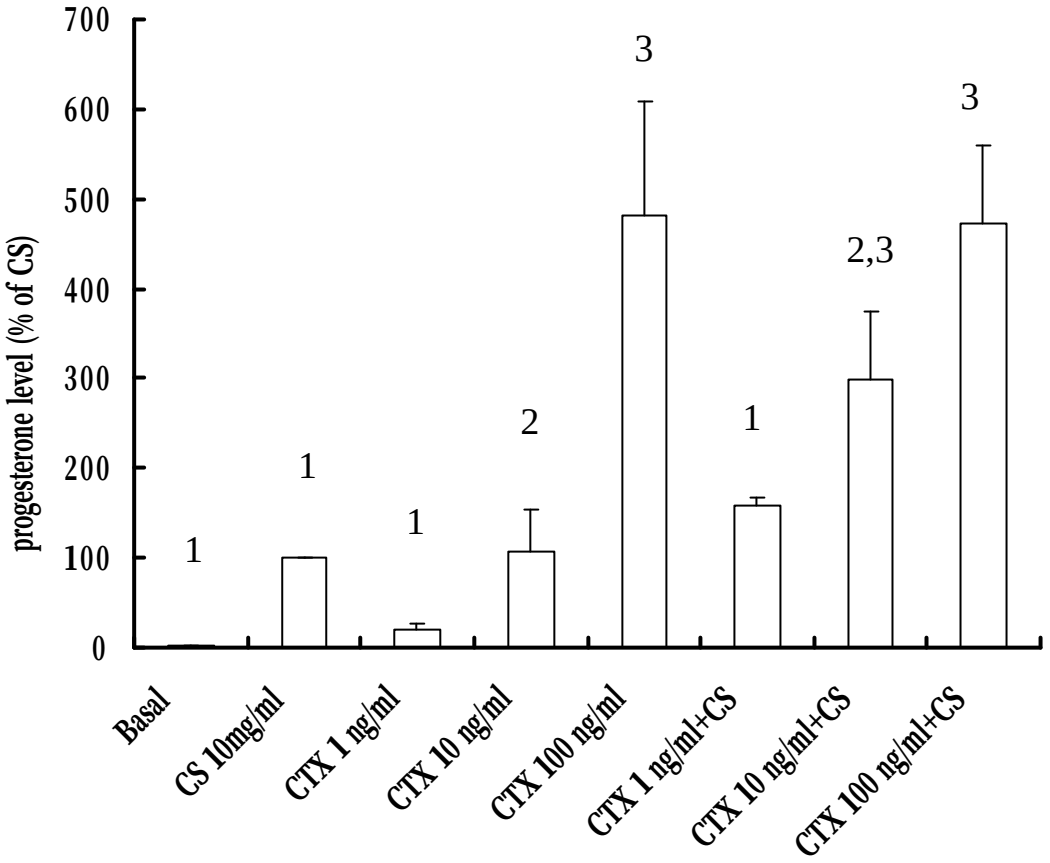

Figure 2. The effects of CS on cholera toxin-treated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of cholera toxin (CTX) (1-100 ng/ml) plus CS (10 mg/ml) for 3 hr incubation. CS enhanced CTX-treated steroidogenesis in additive fashion. Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

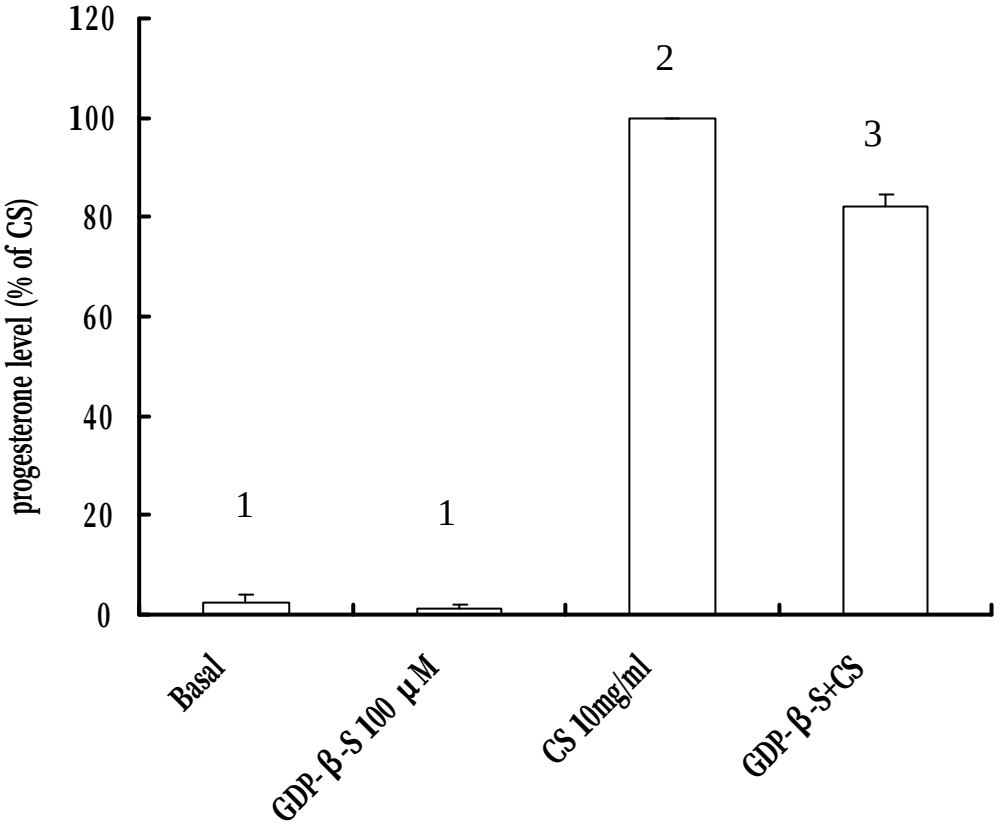

Figure 3. The effect of GDP-β-S on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with GDP- β-S (100 μM) plus CS (10 mg/ml) for 3 hr incubation. GDP- β-S (100 μM) significantly inhibited CS-stimulated progesterone production by 18 ± 2% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

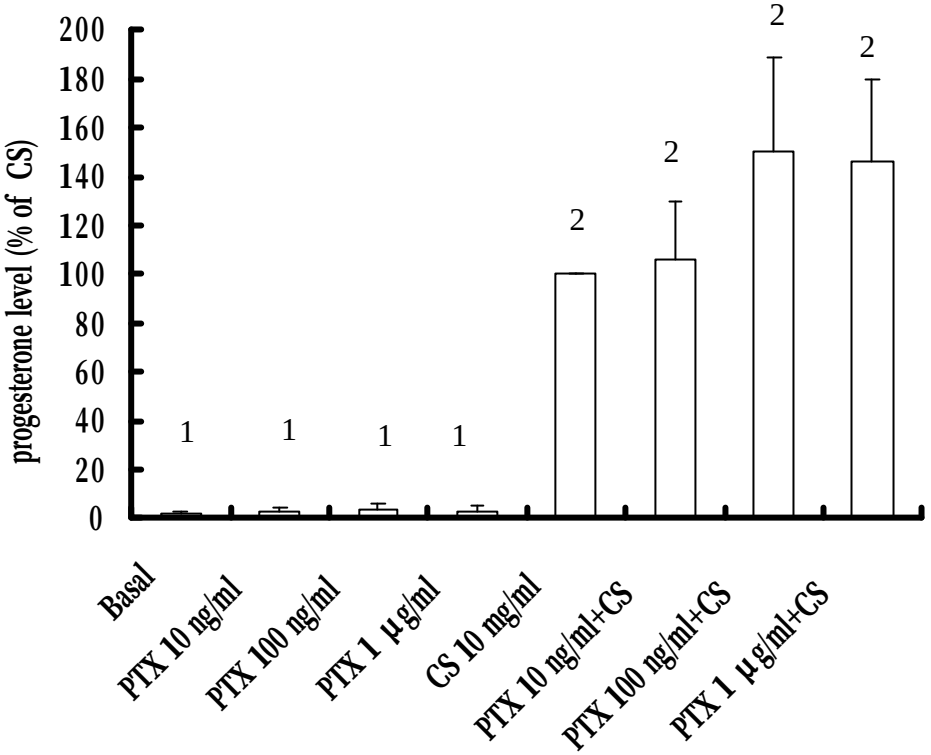

Figure 4. The effects of pertussis toxin (PTX) on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of pertussis toxin (PTX) (10 ng/ml-1 μg/ml) plus CS (10 mg/ml) for 3 hr incubation. PTX (10 ng/ml-1 μg/ml) did not inhibit CS-stimulated steroidogenesis ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

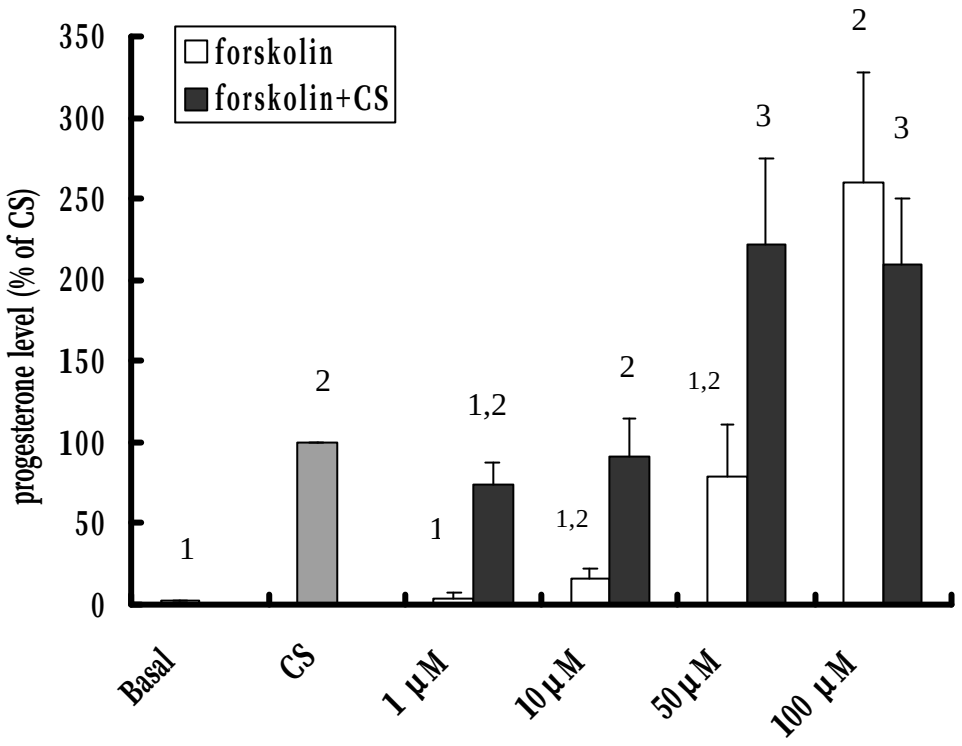

Figure 5. The effects of CS on forskolin-induced steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of forskolin (1-100 μM) plus CS (10 mg/ml) for 3 hr incubation. CS increased forskolin-induced progesterone production in a dosage dependent manner. Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

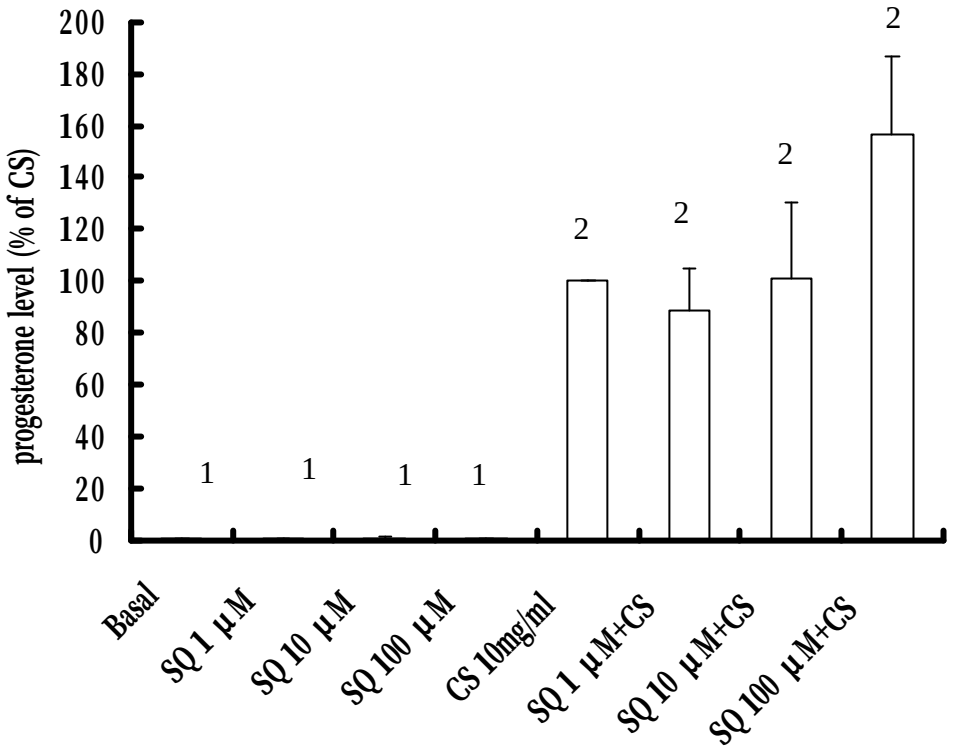

Figure 6. The effects of SQ 22536 on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of SQ 22536 (SQ) (1-100 μM) plus CS (10 mg/ml) for 3 hr incubation. SQ (1-100 μM) did not inhibit CS-stimulated steroidogenesis ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

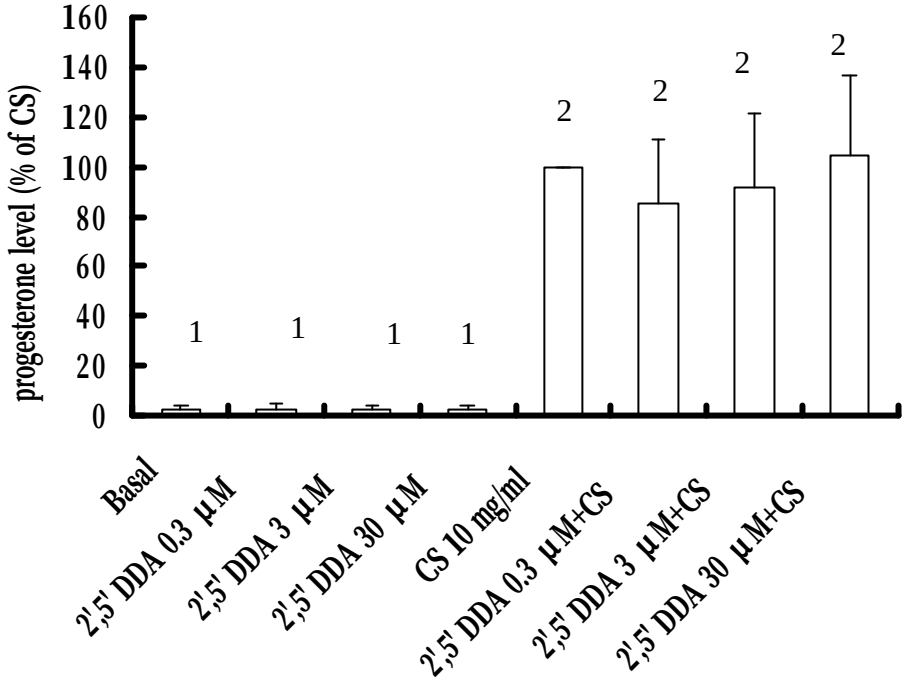

Figure 7. The effects of 2',5' dideoxyadenosine on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of 2',5' dideoxyadenosine (2',5') (0.3-30 μM) plus CS (10 mg/ml) for 3 hr incubation. 2',5' (0.3-30 μM) did not inhibit CS-stimulated steroidogenesis ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

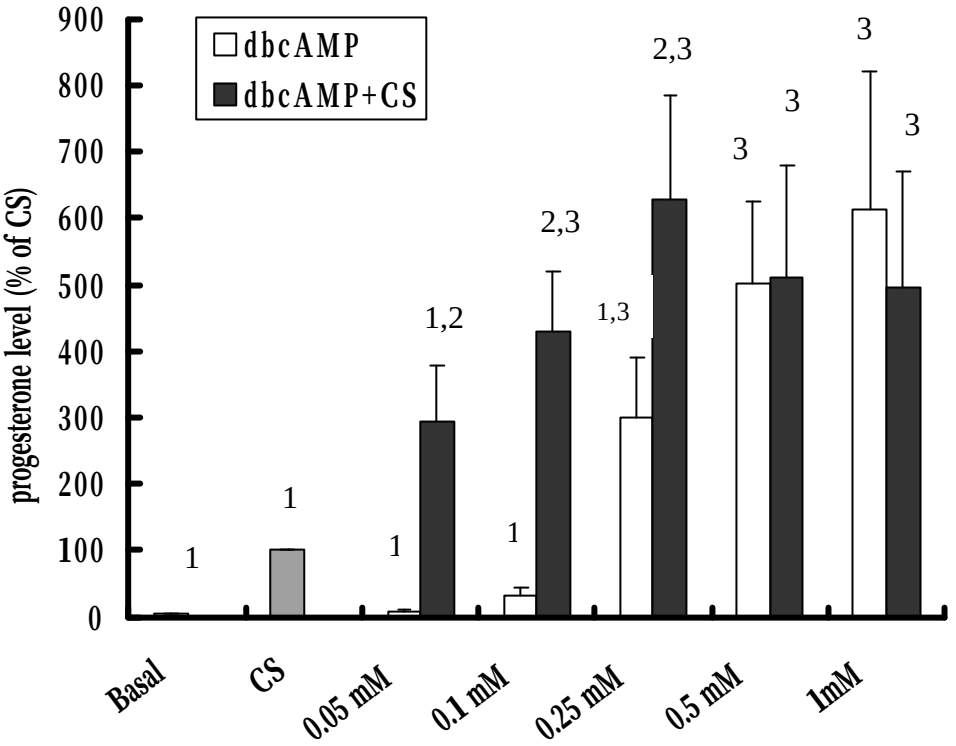

Figure 8. The effects of CS on dbcAMP-induced steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of dbcAMP (0.05-1.0 mM) plus CS (10 mg/ml) for 3 hr incubation. CS increased dbcAMP-induced progesterone production in a dosage dependent manner. Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

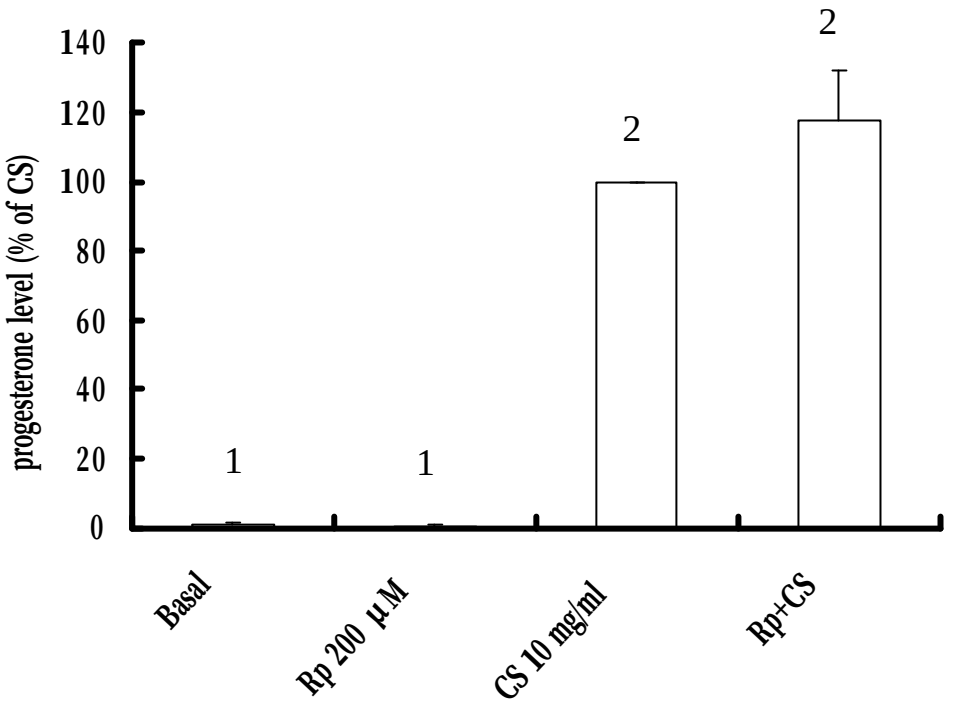

Figure 9. The effect of Rp-cAMPS on CS-stimulated steroidogenesis in MA-10 cells. Cells were pretreated with Rp-cAMPS (Rp) (200 μM) for 30 min. After 30 min, cells were treated without or with Rp (200 μM) plus CS (10 mg/ml) for 3 hr incubation. Rp (200 μM) didn't inhibit CS-stimulated progesterone production ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

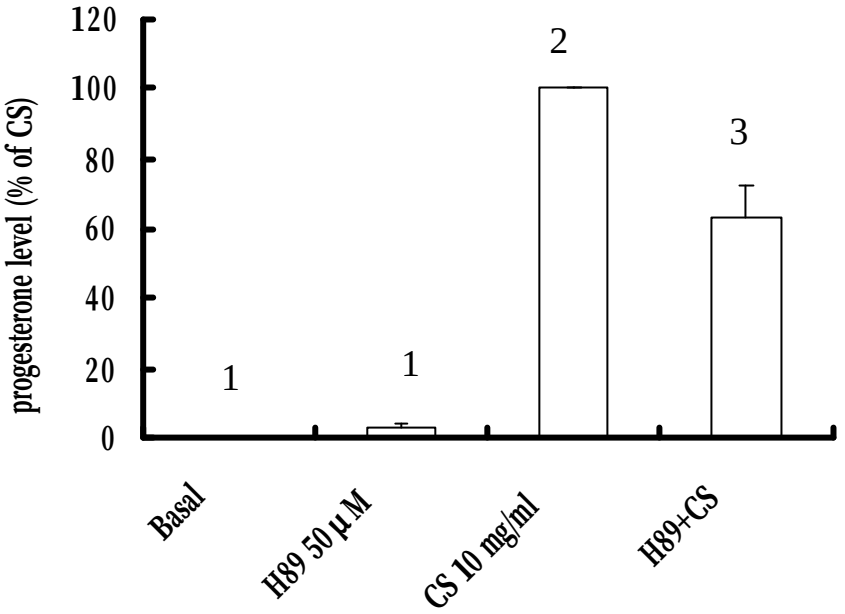

Figure 10. The effect of H89 on CS-stimulated steroidogenesis in MA-10 cells. Cells were pretreated with H89 (50 μM) for 30 min. After 30 min, cells were treated without or with H89 (50 μM) plus CS (10 mg/ml) for 3 hr incubation. H89 (50 μM) inhibited CS-stimulated steroidogenesis by 37 ± 9% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

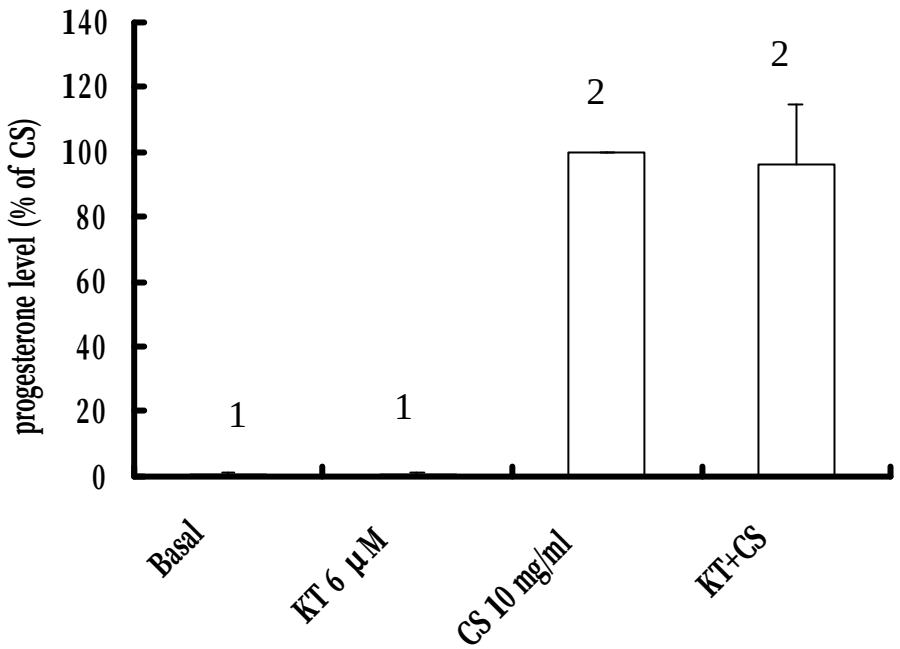

Figure 11. The effect of KT 5720 on CS-stimulated steroidogenesis in MA-10 cells. Cells were pretreated with KT 5720 (KT) (6 μM) for 30 min. After 30 min, cells were treated without or with KT (6 μM) plus CS (10 mg/ml) for 3 hr incubation. KT (6 μM) didn't inhibit CS-stimulated progesterone production ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

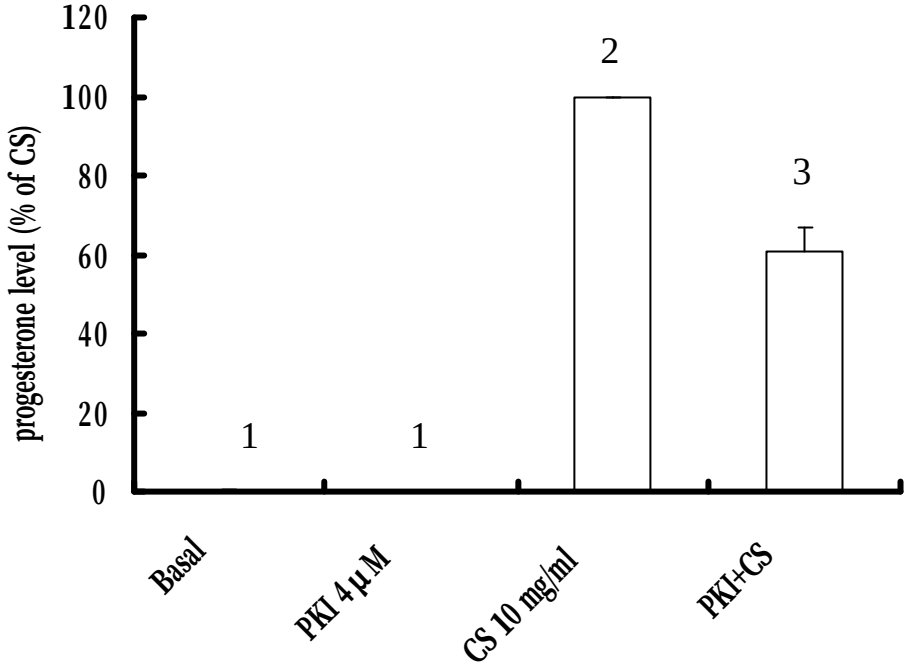

Figure 12. The effect of PKI on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with PKI (4 μM) plus CS (10 mg/ml) for 3 hr incubation. PKI (4 μM) significantly inhibited CS-stimulated steroidogenesis by 40 ± 6% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

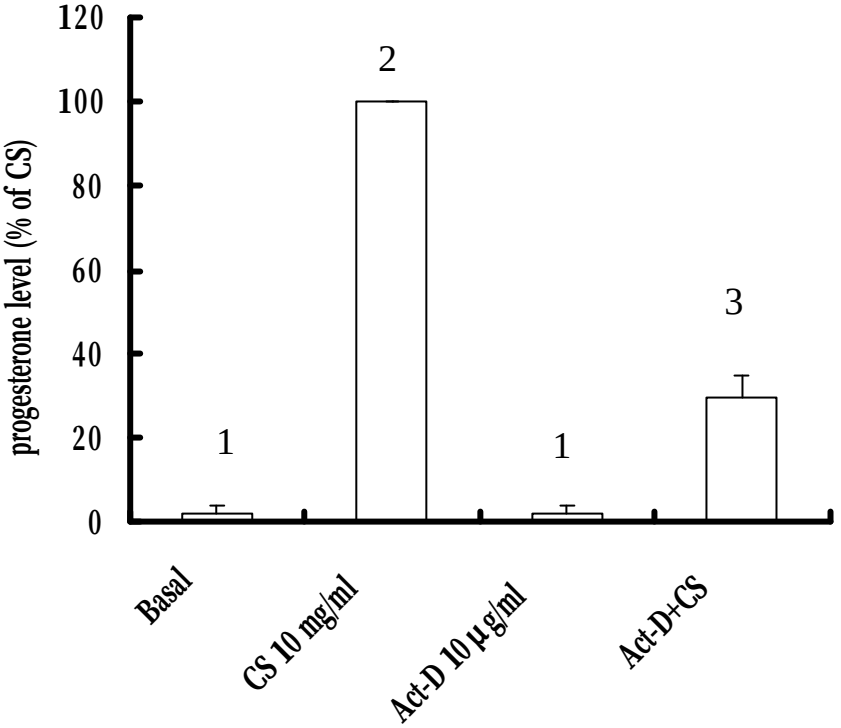

Figure 13. The effect of actinomycin D on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with actinomycin (Act-D) (10 μg/ml) plus CS (10 mg/ml) for 3 hr incubation. Act-D (10 μg/ml) significantly inhibited CS-stimulated steroidogenesis by 71 ± 9% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

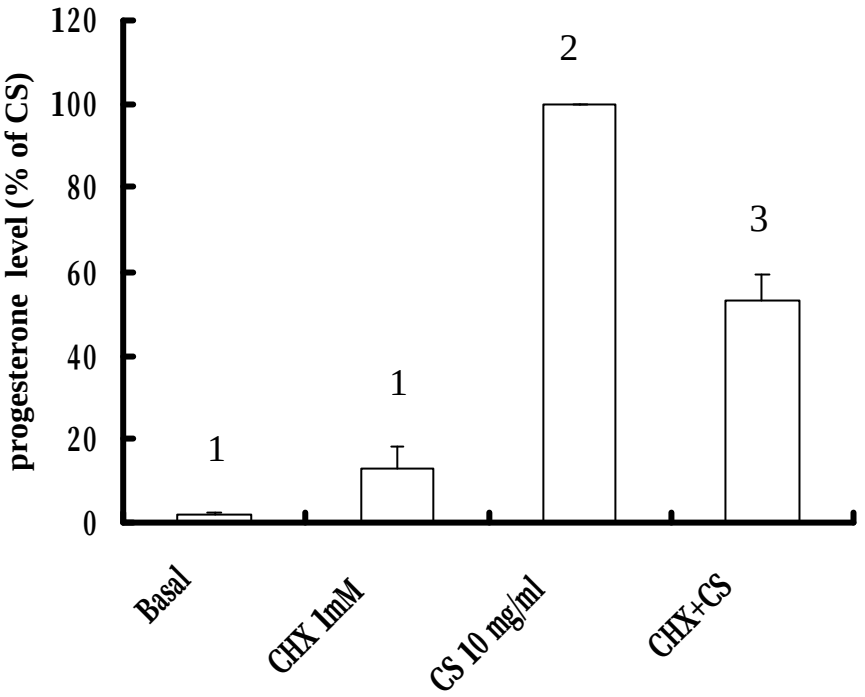

Figure 14. The effect of cycloheximide on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with cycloheximide (CHX) (1 mM) plus CS (10 mg/ml) for 3 hr incubation. CHX (1 mM) inhibited CS-stimulated steroidogenesis by 48 ± 6% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

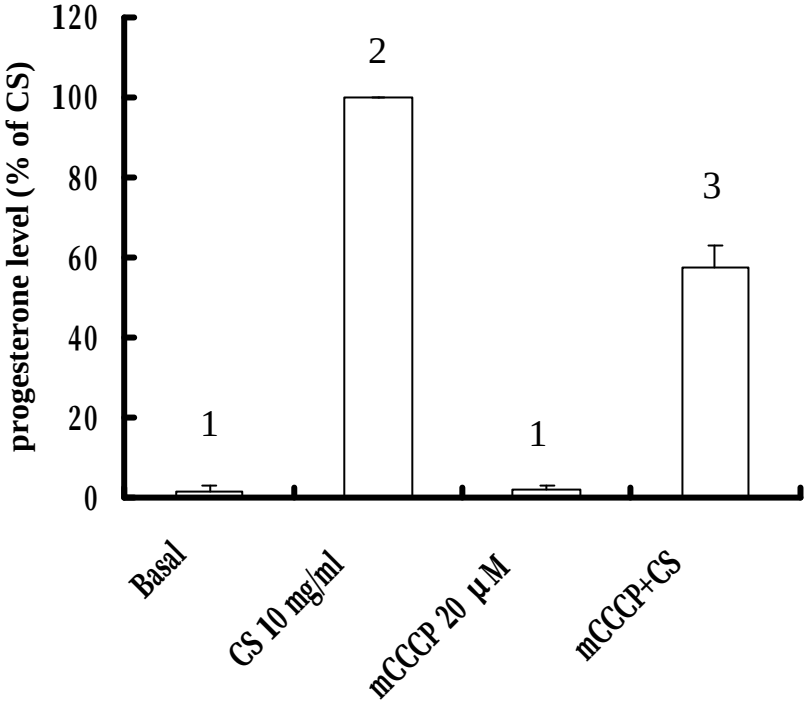

Figure 15. The effect of mCCCP on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with mCCCP (20 μM) plus CS (10 mg/ml) for 3 hr incubation. The mCCCP (20 μM) inhibited CS-stimulated steroidogenesis by 43 ± 5% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

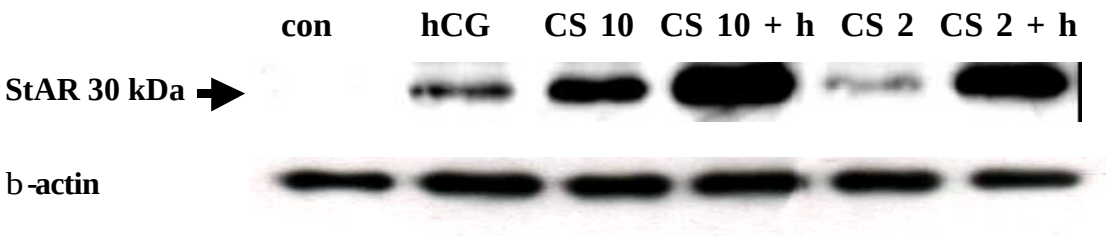

Figure 16. The effects of CS on StAR protein expression in MA-10 cells. The StAR specific bands were detected by Western blot from total protein. Cells were treated with vehicle, hCG 50 ng/ml, CS 10 mg/ml, CS 10 mg/ml plus hCG, CS 2 mg/ml, CS 2mg/ml plus hCG for 3 hr incubation.

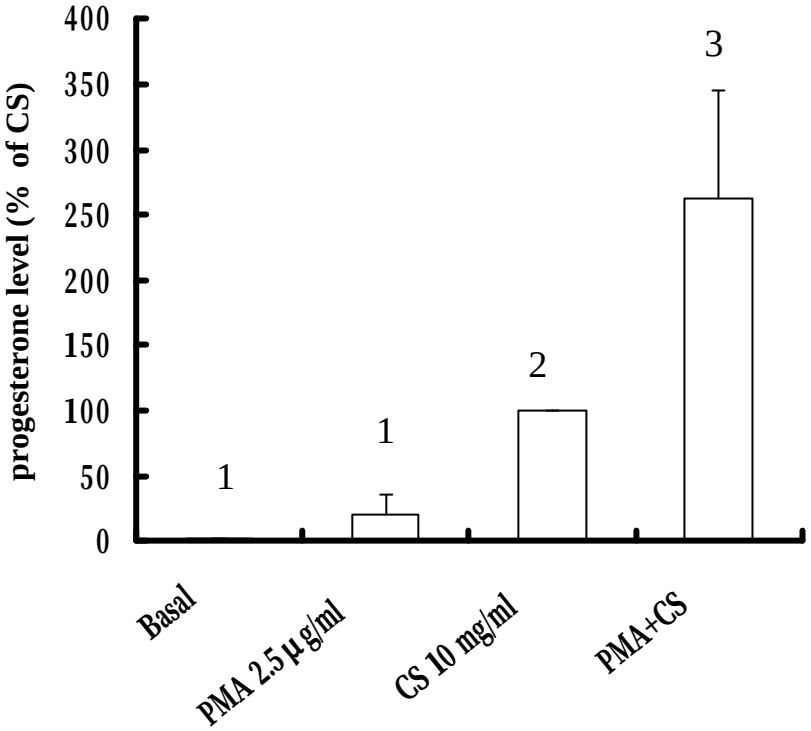

Figure 17. The effect of CS on PMA-treated progesterone production in MA-10 cells. Cells were treated without or with PMA (2.5 μg/ml) plus CS (10 mg/ml) for 3 hr incubation. CS increased PMA-treated progesterone production. PMA (2.5 μg/ml) significantly enhanced progesterone production by 262 ± 82% in the presence of CS ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

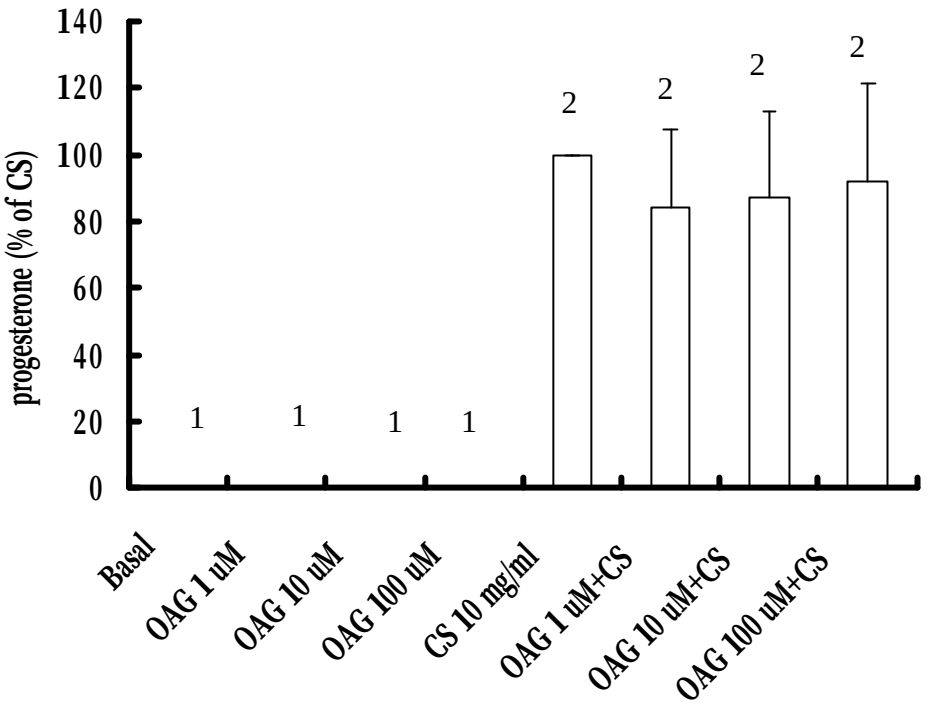

Figure 18. The effects of CS on 1-Oleoyl-2-acetyl-sn-glycerol- treated progesterone production in MA-10 cells. Cells were treated without or with various concentrations of 1-Oleoyl-2-acetyl-sn-glycerol (OAG) (1-100 μM) plus CS (10 mg/ml) for 3 hr incubation. CS didn't affect OAG-treated (1-100 μM) progesterone production ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

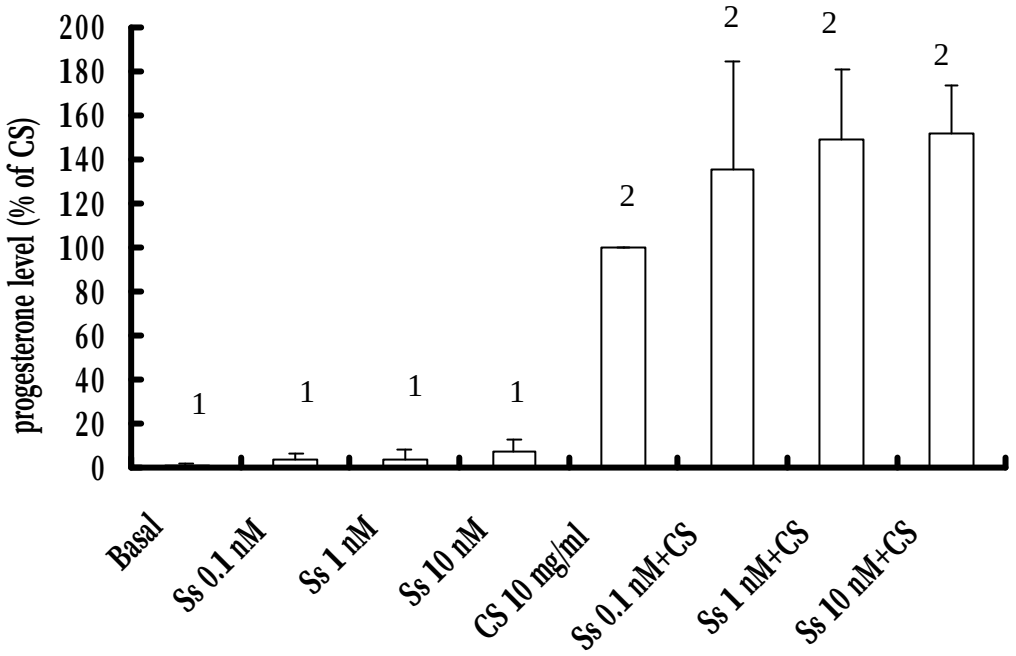

Figure 19. The effects of staurosporine on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of staurosporine (Ss) (0.1-10 nM) plus CS (10 mg/ml) for 3 hr incubation. Ss (0.1-10 nM) didn't inhibit CS-stimulated progesterone production ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

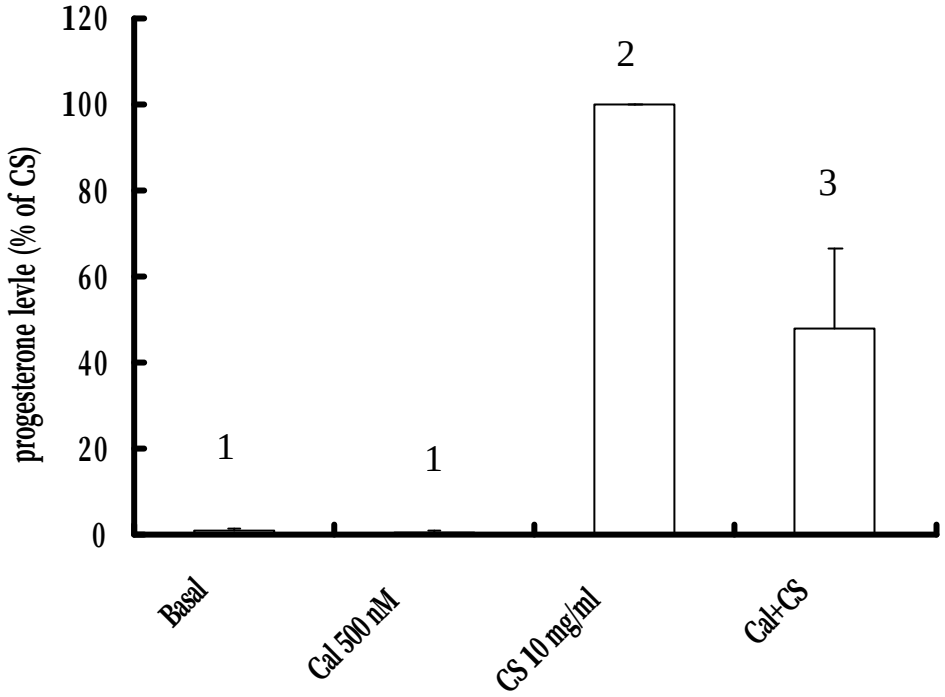

Figure 20. The effect of calphostin C on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with calphostin C (Cal) (500 nM) plus CS (10 mg/ml) for 3 hr incubation. Cal (500 nM) inhibited CS-stimulated progesterone production by 52 ± 18% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

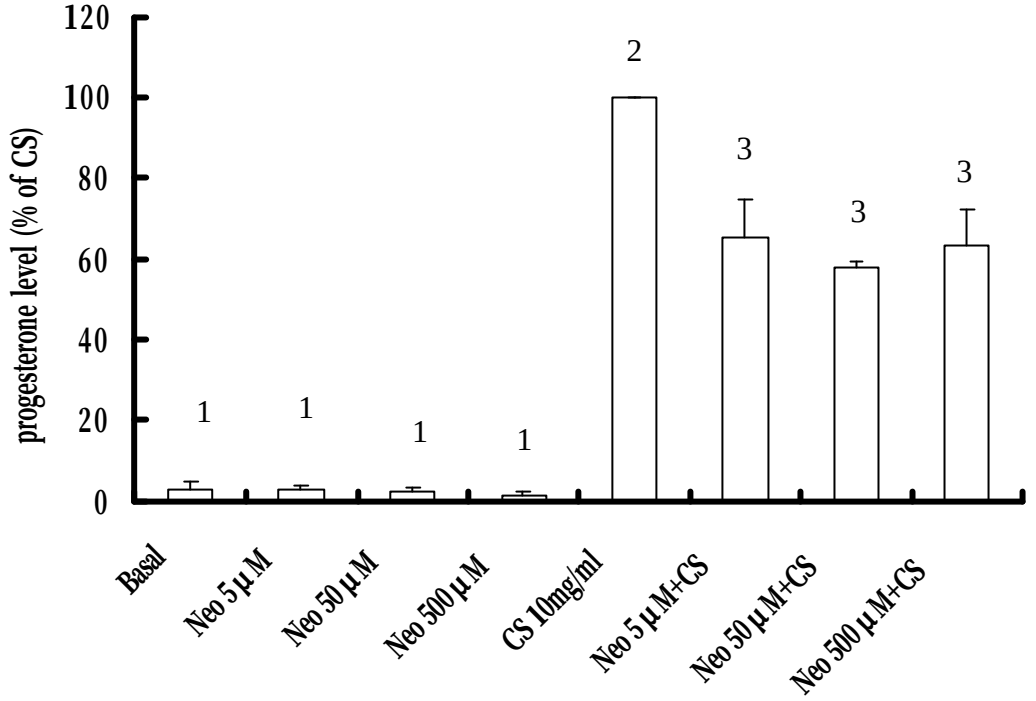

Figure 21. The effects of neomycin sulfate on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of neomycin sulfate (Neo) (5-500 μM) plus CS (10 mg/ml) for 3 hr incubation. Neo (5-500 μM) significantly inhibited CS-stimulated steroidogenesis by 35 ± 9%, 43 ± 1% and 37 ± 9%, respectively ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

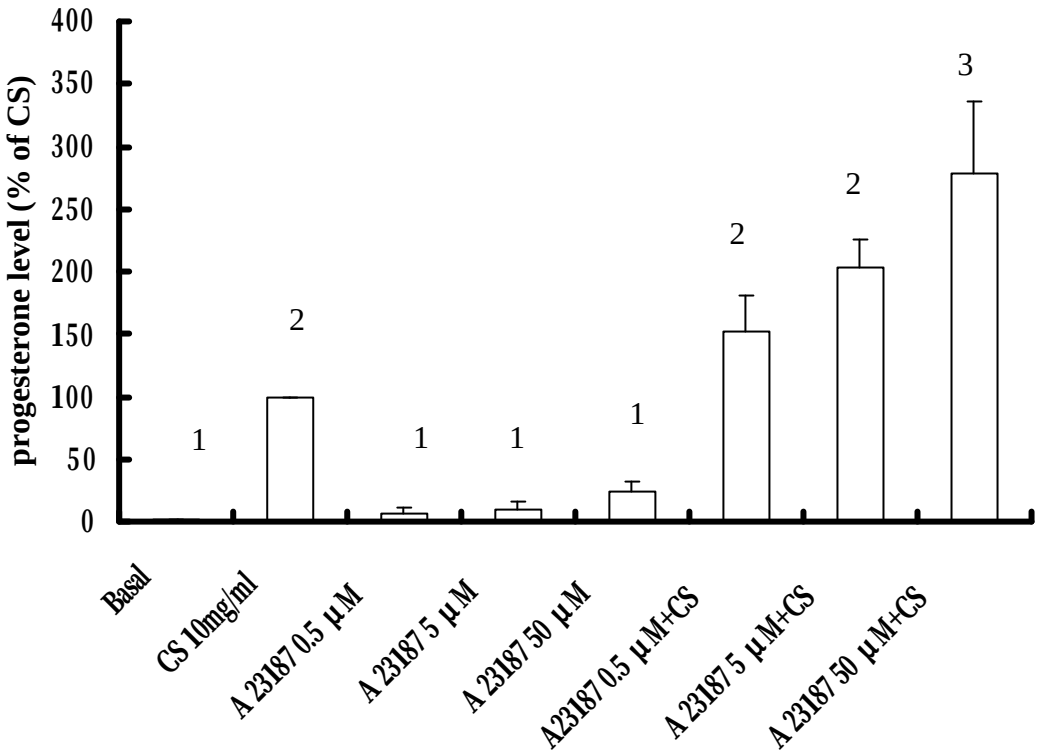

Figure 22. The effects of CS on A23187-treated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of A23187 (0.5-50 μM) plus CS (10 mg/ml) for 3 hr incubation. CS plus A23187 (50 μM) significantly increased progesterone production ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

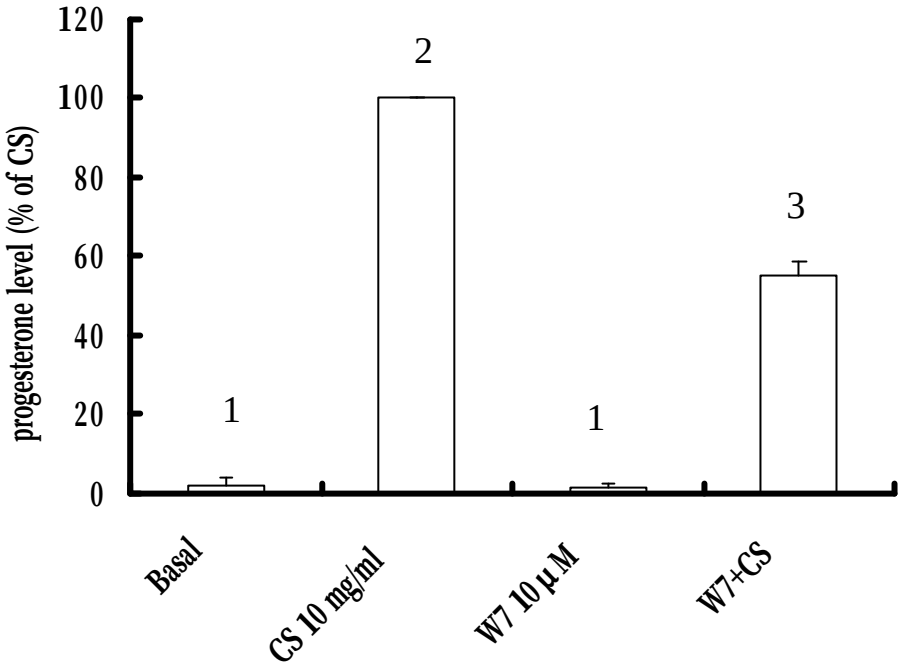

Figure 23. The effect of W7 on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with W7 (10 μM) plus CS (10 mg/ml) for 3 hr incubation. W7 (10 μM) significantly inhibited CS-stimulated progesterone production by 45 ± 3% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

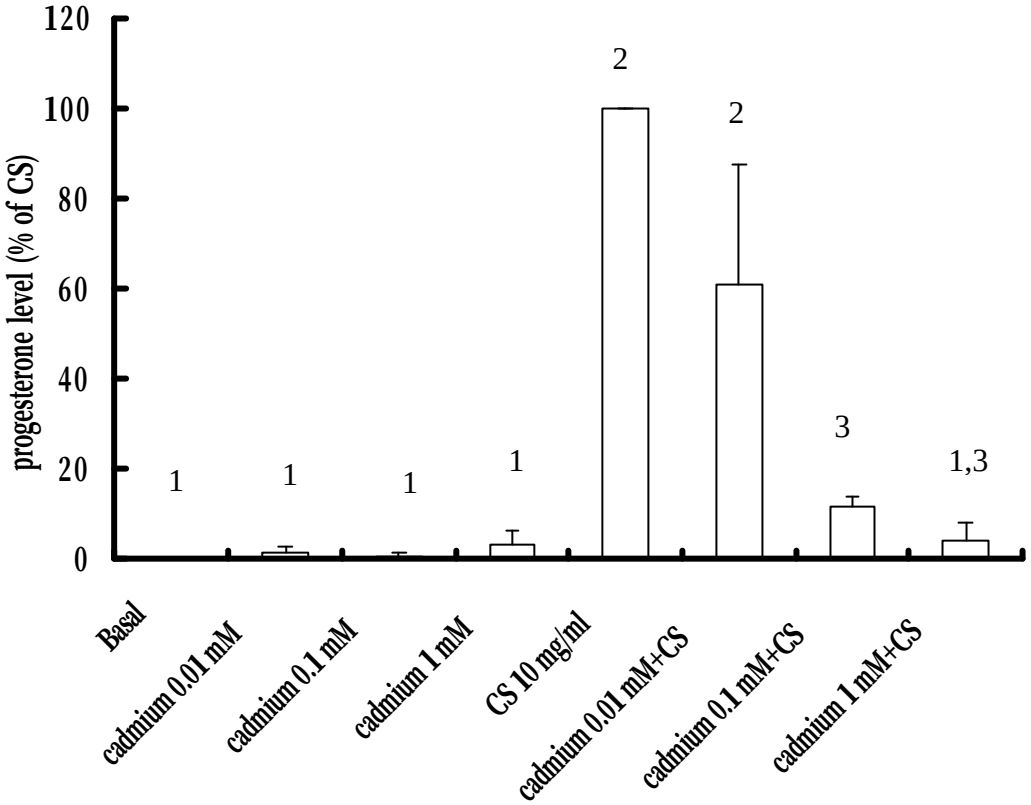

Figure 24. The effects of cadmium on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of cadmium (0.01-1 mM) plus CS (10 mg/ml) for 3 hr incubation. Cadmium (0.1 and 1 mM) inhibited CS-stimulated progesterone production by 88 ± 2% and 96 ± 4%, respectively ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

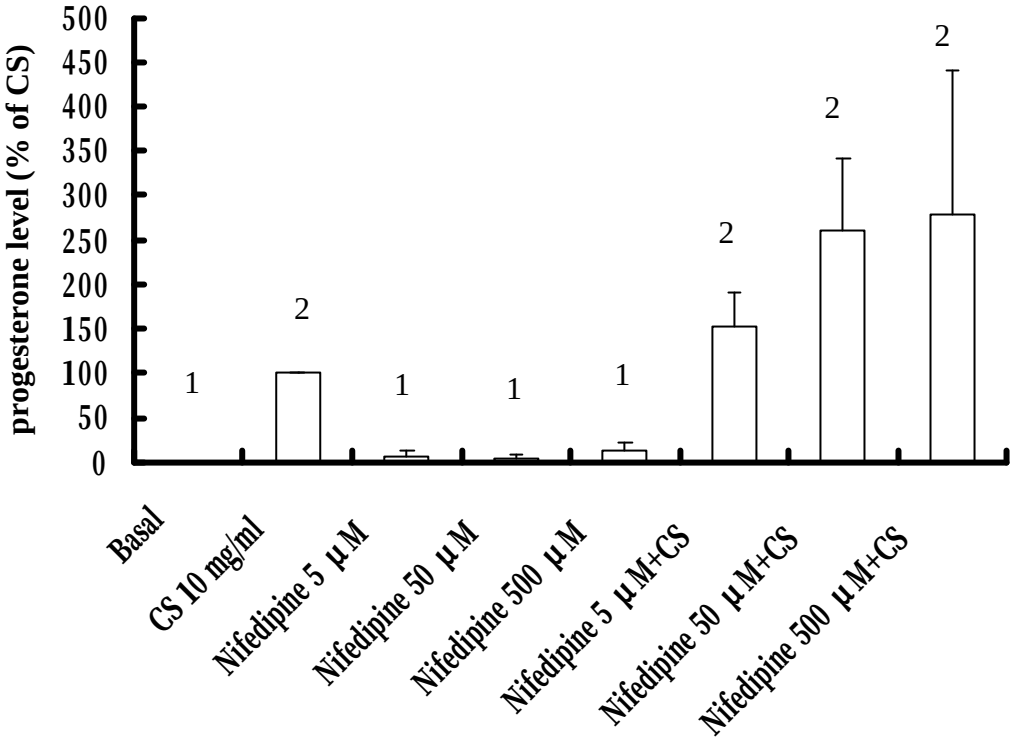

Figure 25. The effects of nifedipine on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of nifedipine (5-500 μM) plus CS (10 mg/ml) for 3 hr incubation. Nifedipine (5-500 μM) didn't inhibit CS-stimulated progesterone production ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

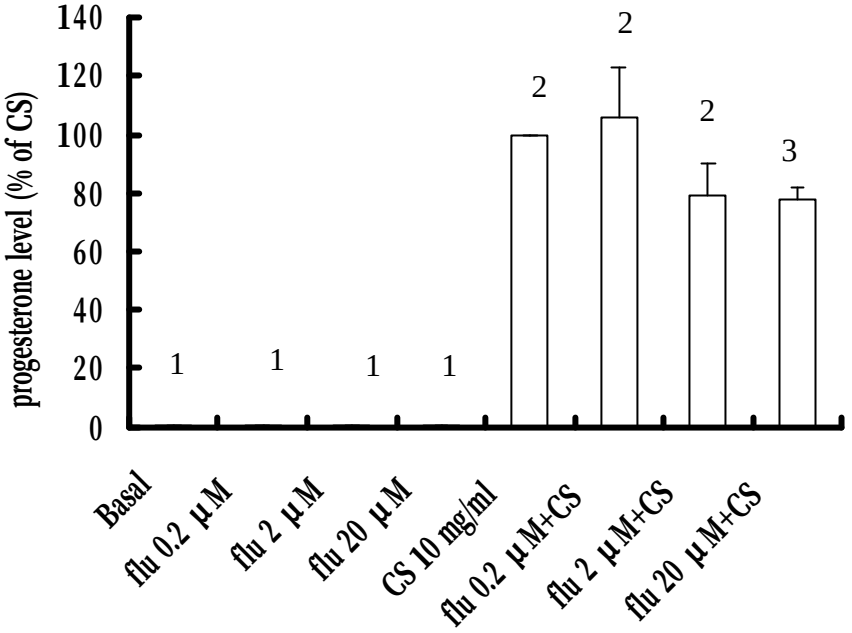

Figure 26. The effects of flunarizine dihydrochloride on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of flunarizine dihydrochloride (flu) (0.2-20 μM) plus CS (10 mg/ml) for 3 hr incubation. flu (20 μM) inhibited CS-stimulated progesterone production by 23 ± 4% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

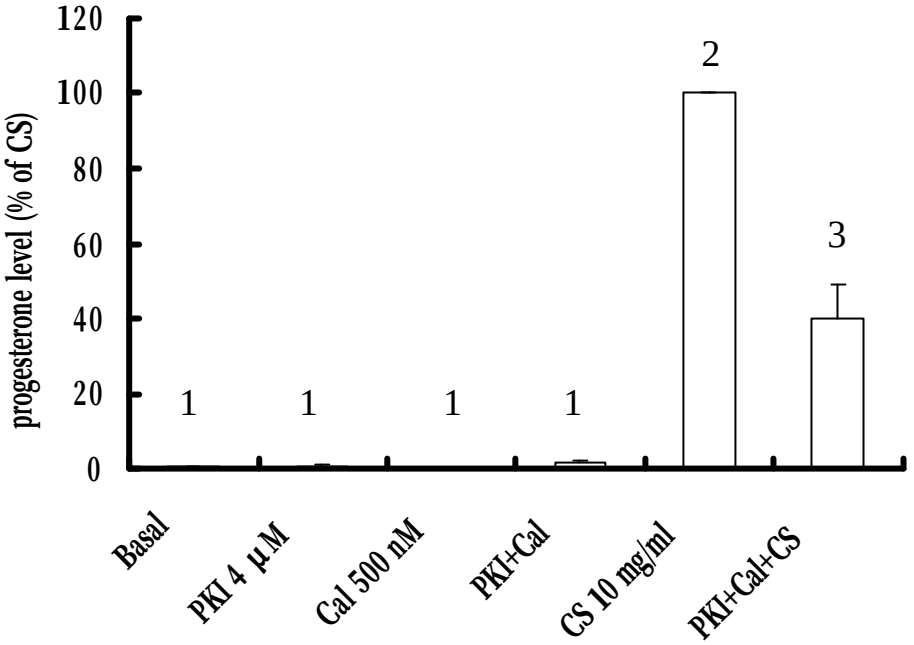

Figure 27. The effect of cotreatment of PKI and calphostin C on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with PKI (4 μM) and calphostin C (500 nM) plus CS (10 mg/ml) for 3 hr incubation. PKI (4 μM) cotreated with calphostin C (500 nM) inhibited CS-stimulated progesterone production by 61 ± 8% ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

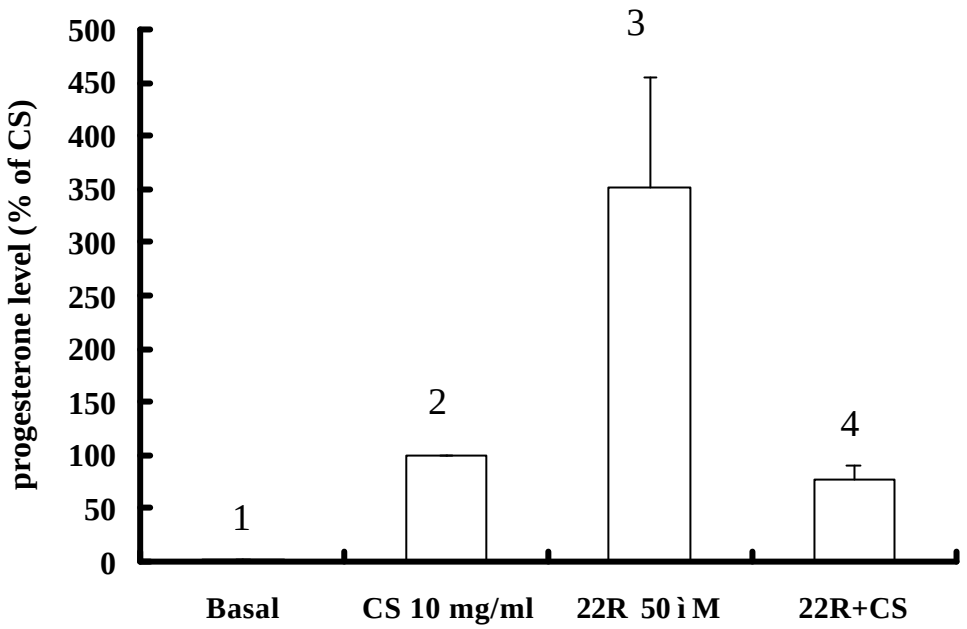

Figure 28. The effect of CS on 22R-hydroxycholesterol- treated steroidogenesis in MA-10 cells. Cells were treated without or with 22R-hydroxycholesterol (22R) (50μ M) plus CS (10 mg/ml) for 3 hr. CS significantly inhibited 22R-treated progesterone production ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

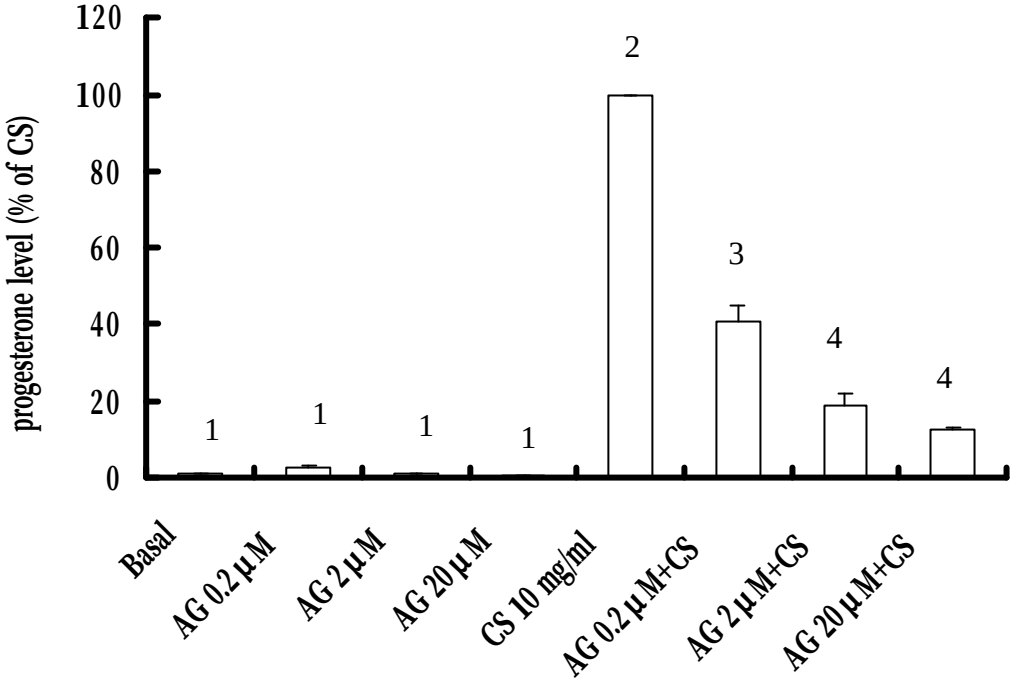

Figure 29. The effect of aminoglutethimide on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of aminoglutethimide (AG) (0.2-20 μM) plus CS (10 mg/ml) for 3 hr incubation. AG (0.2-20 μM) inhibited CS-stimulated progesterone production in a dosage dependent manner and significantly reduced CS-stimulated progesterone production by 60 ± 3%, 72 ± 3% and 88 ± 1%, respectively ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

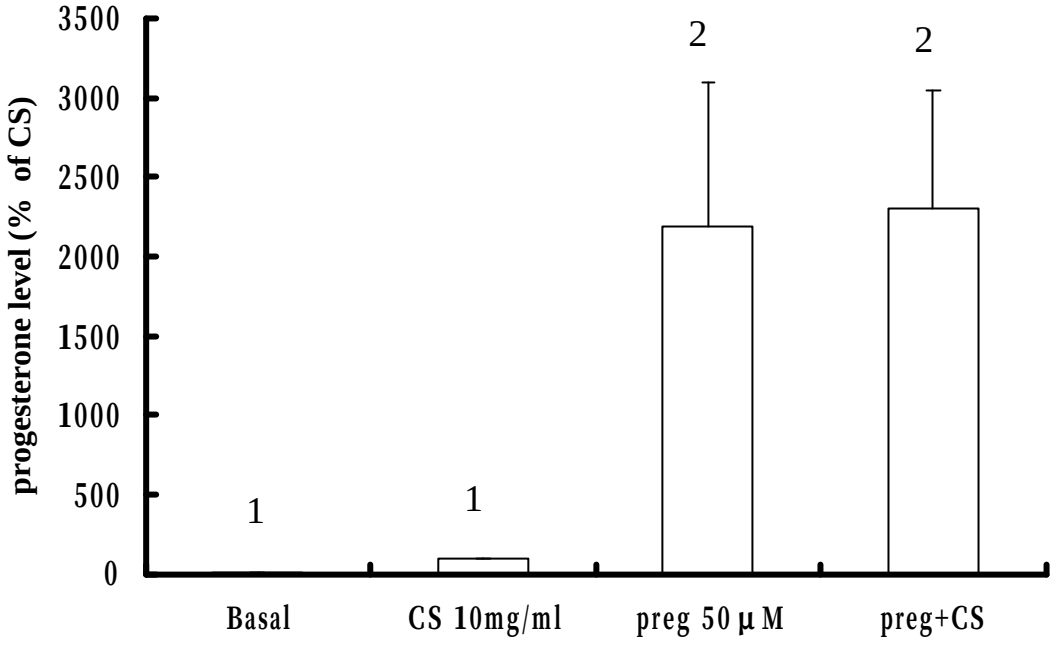

Figure 30. The effect of CS on pregnenolone-treated steroidogenesis in MA-10 cells. Cells were treated without or with pregnenolone (preg) (50μ M) plus CS (10 mg/ml) for 3 hr incubation. CS did not enhance preg-treated progesterone production ($p>0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of more than three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

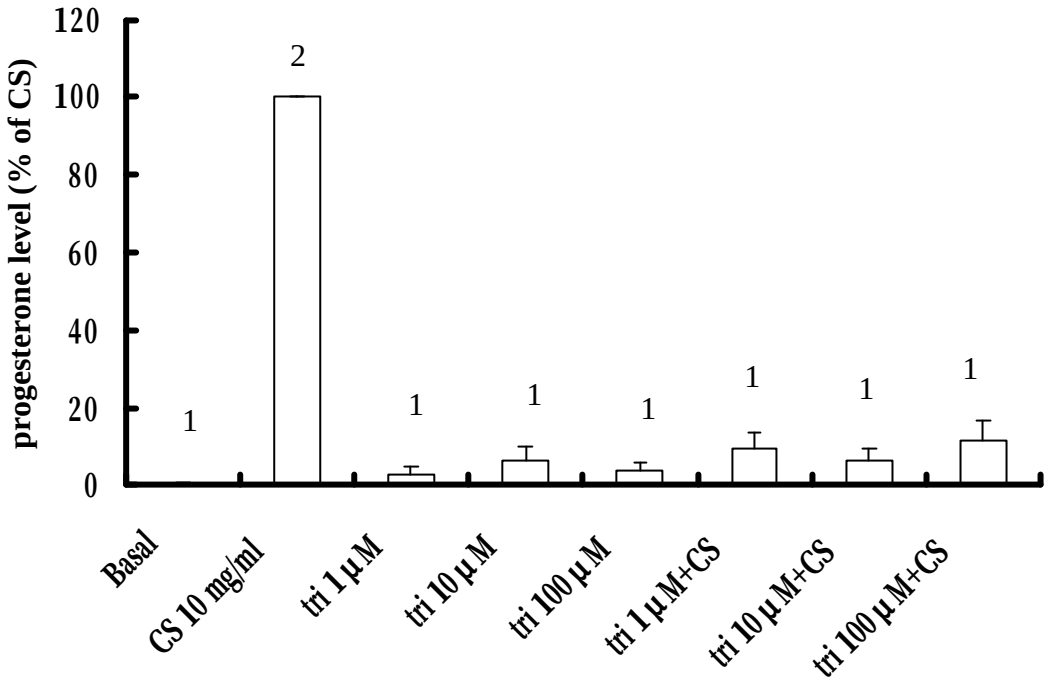

Figure 31. The effect of trilostane on CS-stimulated steroidogenesis in MA-10 cells. Cells were treated without or with various concentrations of trilostane (tri) (1-100 μM) plus CS (10 mg/ml) for 3 hr incubation. tri (1-100 μM) completely inhibited CS-stimulated progesterone production ($p<0.05$). Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean±SEM in percentage of progesterone production of three experiments with triplicates in each treatment. Different number above the column indicates that means are significantly different ($p<0.05$) among each group.

DISSCUSSION

The stimulatory effects of CS on testosterone and progesterone production, respectively, by normal Leydig cells and MA-10 mouse Leydig tumor cells have been demonstrated (Huang et al, 2000; 2001a,b). However, it remains elusive how CS influences MA-10 cell steroidogenesis. In fact, it has been shown that LH/hCG can activate cAMP/PKA pathway to induce steroidogenesis in Leydig cells (Huang et al, 1995). In our present study, the progesterone production in the treatment of CS with hCG was much higher than CS alone treatment ($p<0.05$). Thus, it is possible that CS may directly activate cAMP/PKA pathway for steroidogenesis, or other signal transduction pathways might be involved by CS to enhance LH/hCG-stimulated cAMP signal transduction pathway and then the steroidogenesis. Furthermore, in the present study, we further found that CS (10 mg/ml) might act on both PKA and PKC signal transduction pathway with calcium ion involved to stimulate steroidogenesis. Moreover, the activity of the steroidogenic enzymes, P450scc, might be decreased, whereas the activity of 3β-HSD might not be affected by CS treatment.

Most investigators agree that LH receptor-mediated effects on Leydig cell steroidogenesis are mostly through the activation of the G_s/adenylate cyclase /cAMP/PKA pathway (Ascoli et al, 2002). It is now generally accepted that G protein-coupled receptor (GPCR) can independently activate more than one subfamily of heterotrimeric G proteins

(Gudermann et al, 1996). By binding to LH receptor, LH can activate G_s protein and thus induce the activation of adenylate cyclase. The identification of other G proteins, that are activated by the LH receptor and which of these mediate the activation of the inositol phosphate pathway, is still controversial (Herrlich et al, 1996; Rajagopalan-Gupta et al, 1998).

As shown in figure 3, GDP-β-S (100 μM), a general G protein inhibitor, could significantly inhibit CS-stimulated progesterone production by 18 ± 2% ($p<0.05$). It suggested that CS might affect at least one kind of G proteins in stimulating MA-10 cell steroidogenesis. Based on these results, we tried to find which G protein would be activated by CS treatment in MA-10 cells. As shown in figure 2, CS could enhance cholera toxin-treated progesterone production in a dosage dependent manner. However, figure 4 illustrated that G_i protein inhibitor, pertussis toxin (10 ng/ml-1 μg/ml), didn't block CS-stimulated steroidogenesis ($p>0.05$). Therefore, our results suggested that CS might affect G_s, but not G_i, for CS-stimulated steroidogenesis in MA-10 cells.

At least nine closely related isoforms of adenylate cyclases have been identified (AC1 through AC9). These proteins share several features such as the activation of G_s protein by α subunit, the activation by the diterpene forskolin, and the inhibition by a class of adenosine analogues known as P-site inhibitors (Tesmer et al, 1997; Hurley, 1998; Hanoune and Defer, 2001). Our results demonstrated that adenylate cyclase (AC) inhibitors, SQ 22536 and 2',5' dideoxyadenosine, didn't block the

stimulatory effects of CS. These results suggested that CS might not affect AC activity (Fig. 5, 6, 7) in MA-10 cell steroidogenesis. However, the forskolin-induced progesterone production was increased significantly by CS when forskolin is 50 μM, suggesting that CS might enhance cAMP-PKA pathway to affect MA-10 cell steroidogenesis.

In the present study, we used several PKA inhibitors to determine if PKA pathway was involved in CS-stimulated steroidogenesis. Cyclic AMP-dependent protein kinase (PKA) is a holoenzyme consisting of regulatory (R) and catalytic (C) subunits. Activation of PKA occurs when four molecules of cAMP bind to the R subunit dimer, two on each subunit, in a positive cooperative fashion. When all cAMP binding sites are occupied with R subunit, R and C subunits will dissociate (Mathew and Van holde, 1992; Skalhegg and Tasken, 1997). H89 and KT5720 are PKA inhibitors which abolish PKA activity by blocking the ATP binding site of PKA to interfere with the phosphorylation process of PKA (Wang et al, 2001; Sirotkin and Makarevich, 1999). The present results demonstrated that H89, but not KT5720, significantly blocked CS-stimulated progesterone production by 37 ± 9% ($p<0.05$). Although ATP-site directed inhibitors such as H89 or KT5720 are similar complexes in organic compositions, their efficacy or the side effects of their metabolites might be different in influencing PKA function. (Yuan and Bers, 1995; Davies et al, 2000). Thus, it will be necessary to further evaluate how KT5720 (6 μM) didn't inhibit CS-stimulated progesterone production in MA-10 cells. However, PKI could significantly abolish CS-stimulated steroidogenesis by 40 ± 6% ($p<0.05$). PKI is a

pseudo-substrate of PKA, which binds to the catalytic subunit of PKA and then blocks PKA activation (Pereira et al, 1987; Walsh and Glass, 1991). Indeed, Rp-cAMPS, a cAMP antagonist that competes with intracellular cAMP to prevent PKA holoenzyme from dissociation, was also used to determine if CS would affect PKA activity. Rp-cAMPS (200 μM) didn't block CS-stimulated steroidogenesis, which indicated that the binding of cAMP to PKA affected by CS in MA-10 cells might not be essential. Taken together, the prevention of CS-stimulated progesterone production by PKA inhibitors suggested that the effects of CS on MA-10 cells were probably mediated by the cAMP/PKA system, and that CS might affect the process of phosphorylation in PKA.

Other signal transduction systems have also been shown to be involved in regulating steroidogenesis, including protein kinase C (PKC) and calcium-related pathways. It has been demonstrated that corticosterone production by cultured adrenocortical cells is increased by treatment with water-soluble extract of CS (WECS) and calphostin C, a PKC inhibitor that blocks the effect of WECS in steroidogenesis. These results suggested that the activation of PKC is responsible for WECS-stimulated steroidogenesis (Wang et al, 1998). Our present results also demonstrated that PKC pathway participated in CS-stimulated steroidogenesis in MA-10 cells. Lin (1985) suggested that phorbol ester (PMA) could directly activate Ca^{2+}/phospholipids-dependent protein kinase (PKC) and increase testosterone production. In present study, PMA enhanced CS-stimulated progesterone production, and calphostin C abolished the stimulatory effect of CS in steroidogenesis. These data were

consistent with the observations by Wang et al. (1998). We also found that CS-stimulated steroidogenesis could be blocked by neomycin sulfate, a PLC inhibitor. According to these results (Fig. 20-24), not only PKC pathway was activated by CS treatment but PLC enzyme activity was stimulated by CS in steroidogenesis as well. MA-10 cells do express an isoform of PLC that can be activated by $G_{\alpha q}$ (Hirakawa et al, 2002). Previous studies illustrate that PLC activation requires high concentrations of LH/hCG. (Gudermann et al, 1992). The present study suggested that CS (10 mg/ml) may up-regulate LH receptors or function as LH analogue to activate inositol phosphate/diacylglycerol pathway to stimulate steroidogenesis. However, PKC pathway inhibitors (i.e., staurosporine) or diacylglycerol analogue, OAG didn't have any effect on CS-stimulated steroidogenesis in MA-10 cells, which will need more experiments to further examine. Based on the above data, PKC pathway was activated by CS in MA-10 cell steroidogenesis. In our present data, PKA inhibitors, H89 or PKI, blocked CS-stimulated progesterone production by 30-40% and PKC inhibitor, calphostin C, also blocked CS-stimulated effect by 30-40%. Furthermore, our experiments illustrated that cotreatment of PKI and calphostin C would abolish the stimulatory effect of CS by 50-80% only. This strongly suggested that some other pathways, beside PKA and PKC, might be involved to activate MA-10 cells by CS.

Calcium influx has been reported to activate the PKC system (Berridge et al, 2000; Nishizuka, 1992; Brown and Macleod, 2001). It has been illustrated that calcium plays a pivotal role in steroidogenesis

(Adebanjo et al, 1998; Manna et al, 1999; Liu et al, 2000). In adult rat Leydig cells, LH-stimulated testosterone production is inhibited in the absence of extracellular calcium (Kumar et al, 1994). Our data in MA-10 cells demonstrated that the increasing concentration of cytosolic calcium promote CS-stimulated progesterone production, and the blockage of extracellular calcium influx could inhibit progesterone production by using cadmium, a non-specific calcium channel blocker. In addition, CS-stimulated steroidogenesis could be inhibited by 45 ± 3% ($p<0.05$) by W7, a calmodulin antagonist. Moreover, our results also supported that CS-stimulated steroidogenesis might go through T-type calcium channel, but not L-typed. These data suggested that calcium played some roles in CS-stimulated steroidogenesis.

It has been reported that de novo protein synthesis is required for steroidogenesis (Stocco, 1997; Clark et al, 1997). In our present study, the stimulatory effects of CS in steroidogenesis were prevented by actinomycin D and cycloheximide. The results indicated that CS would induce new protein synthesis for steroidogenesis in MA-10 cells.

The protonophore of electron transport chain, mCCCP, acts by disrupting the electrochemical gradient across the inner mitochondrial membrane and thus blocks the import of mitochondrial preproteins into the mitochondria (Jaussi et al, 1982; Tzagolff, 1982). Miller and colleagues subject StAR protein to limit proteolysis at different pH values, and find that the molecule is altered as the pH decreases. They also show that StAR can form a molten globule only in the range of pH 3.5-4.0 and

propose that if the mitochondrial microenvironment is acidic, StAR molecule may undergo a conformational shift and folded ideally suit to transport lipids, or bind cholesterol with unit stoichiometry (Bose et al, 1999). More recent evidences indicate that import of StAR protein into mitochondria is not required for cholesterol transfer (Arakane et al, 1996; Wang et al, 1998). However, the intermembrane shuttle mechanism of StAR is not consistent with the notion that StAR acts outside of the outer mitochondrial membrane. Instead, the electrochemical gradient across the mitochondrial membrane is required for StAR-mediated steroidogenesis, which seems to be more consistent with the requirement for protein import. However, the exact mechanism of StAR promotes transfer of intermembrane cholesterol is still inconclusive. Stocco and colleagues (1999) demonstrate that mCCCP inhibits both steroidogenesis and processing of StAR in transfected COS-1 cells. Thus, we have undertaken experiments to define the requirement of electrochemical gradient for CS-stimulated steroidogenesis in MA-10 cells. Our present study demonstrated that the effect of CS was abolished significantly at 43 ± 5% ($p<0.05$) by mCCCP. These results suggested that electrochemical gradient was necessary for CS to stimulate steroidogenesis in MA-10 cells. It implied that CS might induce new protein related to transport cholesterol into mitochondria. Moreover, the expressions of StAR protein were examined in our experiments. The results showed that CS could increase the expression of StAR protein, which provided the powerful evidence that StAR protein was necessary for the stimulatory effects of CS in MA-10 cells.

Steroid synthesis in MA-10 cells involves two key enzymes, P450scc and 3β-HSD. Surprisingly, we found that CS might inhibit P450scc while CS had no effect on 3β-HSD enzyme activity. In consistent with our previous study, CS (10 mg/ml) alone enhanced StAR protein expression (Huang et al, 2001). It is possible that CS may up-regulate StAR protein expression to stimulate more steroid synthesis in MA-10 cells to compensate the inhibitory effect of CS on P450scc activity. It is also possible that CS may contain stimulatory and inhibitory substances to stimulate cAMP/PKA pathway and to inhibit P450scc enzyme, simultaneously and respectively, to influence MA-10 cell steroid synthesis (Huang et al, 2001a). The inconsistence that the activity of P450scc was reduced by CS with increasing steroid production in MA-10 cells will need more experiments to clarify.

In conclusion, the mechanisms of CS on stimulating steroidogenesis in MA-10 mouse Leydig tumor cells might partially go through PKA pathway and partially go through PKC pathway with calcium ion involved. StAR protein also plays an important role in this stimulation.

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