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Effects of a Water-Soluble Extract of *Cordyceps sinensis* on Steroidogenesis and Capsular Morphology of Lipid Droplets in Cultured Rat Adrenocortical Cells

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Abstract *Cordyceps sinensis* contains a factor that stimulates corticosteroid production in the animal model. However, it is not known whether this drug acts directly on the adrenal glands or indirectly via the hypothalamus-pituitary axis. In the present study, we used primary rat adrenal cell cultures to investigate the pharmacological function of a water-soluble extract of *Cordyceps sinensis* (CS) and the signaling pathway involved. Radioimmunoassay of corticosterone indicated that the amount of corticosterone produced by adrenal cells is increased in a positively dose-dependent manner by CS, reaching a maximun at 25 μg/ml. This stimulating effect was seen 1 h after CS treatment and was maintained for up to 24 h. Concomitantly, the lipid droplets in these cells became small and fewer in number. Immunostaining with a monoclonal antibody, A2, a specific marker for the lipid droplet capsule, demonstrated that detachment of the capsule from the lipid droplet occurs in response to CS application and that the period required for decapsulation is inversely related to the concentration of CS applied. The mechanism of CS-induced steroidogenesis is apparently different from that for ACTH, since intracellular cAMP levels were not increased in CS-treated cells. However, combined application with calphostin C, a PKC inhibitor, completely blocked the effect of CS on steroidogenesis, suggesting that activation of PKC may be responsible for the CS-induced steroidogenesis. J. Cell. Biochem. 69:483–489, 1998. © 1998 Wiley-Liss, Inc.

Key words: *Cordyceps sinensis;* adrenal cells; steroidogenesis; signal pathway; PKC

Cordyceps sinensis (CS), a traditional Chinese herbal tonic, is reported to have an antiinflammatory effect and to stimulate the activity of natural killer cells and macrophages [Chen, 1985; Zhang, 1985, Liu et al., 1992; Xu et al., 1992; Kuo et al., 1996]. It also inhibits rejection of organ transplants [Zhu and Yu, 1990] and has been used in combination with traditional drugs in this field [Zhang and Xia, 1990; Zhu and Yu, 1990]. It is possible that the effect of CS on the immune system is mediated via hormonal secretion by the adrenal cortex, since its product, glucocorticoid, is a favorite candidate for immunosupression. In addition, it remains to be clarified whether CS itself or its metabolites stimulate steroid secretion. One of our aims is to study direct effects of CS on primary adrenal cell cultures.

Steroidogenesis in adrenal cells is triggered by the binding of adrenocorticotropin (ACTH) to a membrane receptor, which activates adenylate cyclase and the protein kinase A (PKA) system [Shima et al., 1971; Flockhart and Corbin, 1982; Sharma, 1982]; activation of PKA results in the phosphorylation of cholesterol ester hydralase, a key enzyme in cholesterol hydrolysis [Schimmer, 1995]. Steroidogenesis is also regulated by the protein kinase C (PKC) system [Widmaier and Hall, 1985; Finn et al., 1988; Pelosin et al., 1991], and the signaling pathway involved in CS-mediated steroidogenesis therefore requires further investigation.

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Our laboratory has produced a mouse monoclonal antibody, A2, which specifically labels the proteinaceous capsule of the lipid droplet [Wang and Fong, 1995]. In response to lipolytic stimulus, the capsule becomes detached from the droplet surface, and the decapsulated lipid droplet decreases in size [Fong and Wang, 1997]. Thus, a direct correlation is seen between the capsular morphology of lipid droplets and mobilization of cholesterol.

In the present study, we used primary rat adrenocortical cell cultures to study the effect of CS on steroid production and the capsular morphology of lipid droplets. The signal transduction pathway mediating the effect of CS was also examined.

MATERIALS AND METHODS

Preparation of Water Extracts of *Cordyceps sinensis*

The mycelium of artificially cultured *Cordyceps sinensis* was extracted with water, and the supernatants (water extracts) were collected by centrifugation and then lyophilized. The material was resconstituted using distilled water and its protein concentration measured using the Bio-Rad (Richmond, CA) protein assay kit.

Cell Culture

Rat adrenal glands were isolated, minced, and incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY), containing 1 mg/ml of collagenase, for 30 min at 37°C. After mechanical dispersion with a glass pipette, the dissociated cells were collected by low-speed centrifugation and then resuspended in a mixture of Ham's F12 medium and DMEM (1:1 v/v), supplemented with 1.2 g/l of $NaHCO_3$, 5% horse serum, 2.5% fetal calf serum, and 1% penicillin and streptomycin. Cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 for 2 days.

Immunofluorescence Microscopy

Adrenocortical cells, grown on coverslips, were fixed in 0.15% glutaraldehyde (in phosphate buffered saline [PBS]) for 5 min and then treated with $NaBH_4$ (1 mg/ml in PBS) for 30 min. After washes in PBS, the cells were reacted for 1 h at 37°C with mouse monoclonal antibody A2 (1:200 dilution of ascites), which labels the capsule of the lipid droplet [Wang and Fong, 1995]. They were then washed with PBS and reacted with FITC-conjugated goat antimouse IgG (H+L) (Sigma, St. Louis, MO) for 1 h at 37°C. After an extensive PBS wash, the cells were mounted and examined using a Leica fluorescence microscope (Polyvar, Leica, Vienna).

Corticosterone Radioimmunoassay

Day 3 adrenal cultures were used in the following experiments. After drug treatment, 5 ?l of the culture medium was diluted (1:100 v/v)

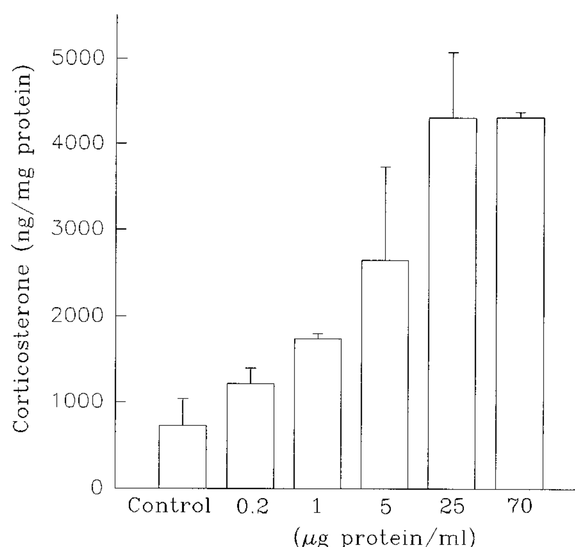

Fig. 1. Dose-response for CS extract on steroidogenesis in rat adrenal cells. Production of corticosterone by the cells shows a positive correlation with the dose of CS extract used between 0.2 and 25 μg/ml. Values are the mean ± SD, n = 3.

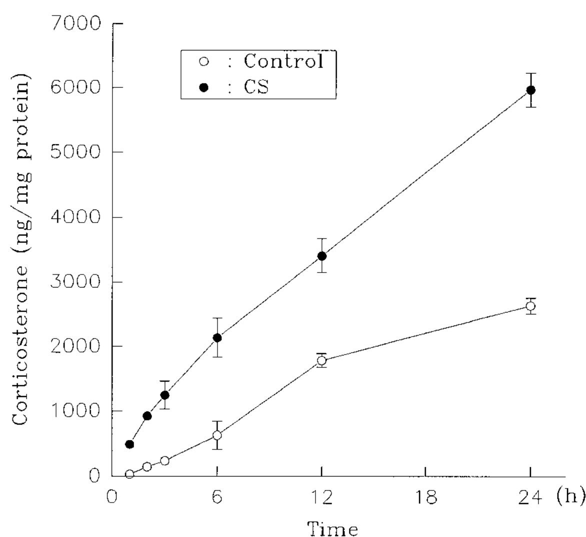

Fig. 2. Time-course study of steroidogenesis induced by CS extract. CS extract (25 μg/ml) increases corticosterone production within 1 h of treatment, and this effect is maintained for up to 24 h. Values are the mean ± SD, n = 3.

with the assay buffer (0.05 M Tris-HCl, pH 8.0, containing 0.1 M NaCl, 0.1% NaN_3, and 0.1% BSA) before addition to the assay tube containing diluted anticorticosterone antiserum (C-8784; Sigma). After 20 min at 37°C, 100 ?l of ^3H-labeled corticosterone (10,000 cpm in assay buffer) was added to each assay tube, and incubation continued for 1 h at 37°C and then for 1 h at 4°C. The free hormones were then adsorbed on 200 μl of dextran-coated charcoal (0.5% dextran and 1.25% charcoal in assay buffer) for 10 min and the bound soluble hormones separated by centrifugation at 13,000 rpm for 10 min. Then 0.7 ml of the supernatant was transferred to a counting vial containing 3 ml of counting solution (Ecoscient H) before counting in a β-counter (LS600IC; Beckman, Fullerton, CA) for 1 min. A standard curve was established using corticosterone standard (Sigma).

cAMP Assay

Three 35 mm dishes of cultures (10^6 cells/dish) were used for each experiment. Drugs (0.01 IU of ACTH, 25 μg/ml of CS, or 500 nM calphostin C) were added to the cultures for 30 min to 6 h in the presence of 500 μM 3-isobutyl-1-methylxanthine (Sigma) to inhibit phosphodiesterase activity. The culture medium was then

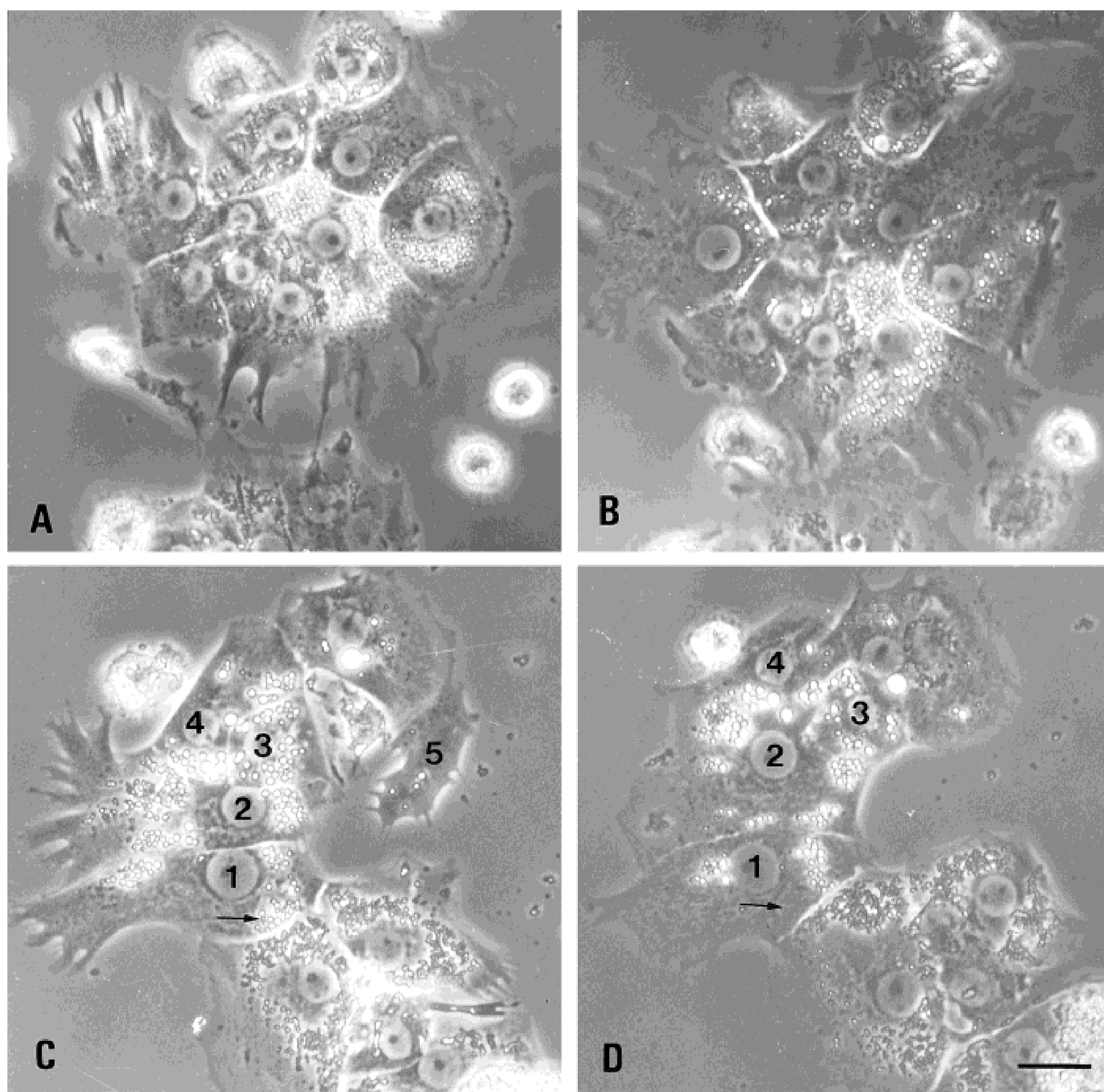

Fig. 3. Effect of CS extract on the morphology of rat adrenal cells. **A,B:** Control cells at 0 h and 12 h. **C,D:** Cells at 0 h and 12 h after CS (25 μg/ml) treatment. Cells are flattened and contain many lipid droplets, cellular processes are retracted (cells 1, 2, and 4 in D) or detached (cell 5 in C) from the substratum, and the total number of lipid droplets in cells 1, 2, and 3 is decreased (arrows). Scale bar = 25 μm.

aspirated off, and the cells were washed with PBS and then treated overnight with 0.5 ml of 0.01 N HCl to extract the cAMP. The supernatant from one dish was collected, neutralized with 5 μl of 1 N NaOH, and centrifuged at 13,000 rpm for 5 min; 10 μl of the supernatant was used to measure intracellular cAMP levels using the Amersham (Arlington Heights, IL) [H^3]-cAMP assay system, based on competition between intracellular cAMP and [H^3]-cAMP for the binding sites of a limited amount of cAMP-binding protein. A standard curve for cAMP was established using a cAMP standard.

RESULTS

Cultured rat adrenal cells produce corticosterone [O'Hare and Neville, 1973]. Addition of CS to the cultures resulted in increased corticosterone levels; this was dose-dependent (Fig. 1), with the saturation dose being 25 μg/ml. Since CS does not contain any corticosterone, the increased corticosterone was produced by the cells. Figure 2 shows a time-course study of control and CS-stimulated steroidogenesis. In control cells, a steady basal production of corticosterone was seen. Corticosterone production was slightly increased 1 h after CS application and increased steadily for 24 h (Fig. 2).

The adrenal cells showed some morphological changes following CS treatment. Control cells were polygonal in shape and contained many lipid droplets. On addition of CS, the cell processes retracted, some cells even becoming detached from the substratum, especially at a high dose, while the number and size of lipid droplets decreased (Fig. 3). The capule morphology of the lipid droplets was further examined by immunofluorescence staining with antibody A2. In control cells, each lipid droplet was surrounded by a bright intact capsule (Fig. 4), while after CS treatment the capsule became discontinuous and stained only weakly (Fig. 5). The time required for decapsulation was dependent on the concentration of CS used (2 h at 25 μg/ml and 7 h at 4 μg/ml).

We then studied the signaling pathway involved in CS-induced steroidogenesis. In contrast to the high level of cAMP induced by ACTH, CS treatment did not produce any change in intracellular cAMP levels (Fig. 6), nor did the CS preparation contain any cAMP activity. In rat adrenal cells, another pathway involved in steroidogenesis is the PKC system [Widmaier and Hall, 1985; Finn et al., 1988; Pelosin et al., 1991]. Calphostin C, a PKC inhibitor, decreased the basal steroid production of the adrenal cells (Fig. 7) and, when applied together with CS, completely inhibited the stimulatory effect of CS on steroidogenesis (Fig. 7). In a time-course study, this inhibitory effect on CS activity was seen as early as 90 min after treatment and lasted for at least 6 h, suggesting that once the PKC activity of the cells is inhibited, the stimulatory effect of CS is abolished.

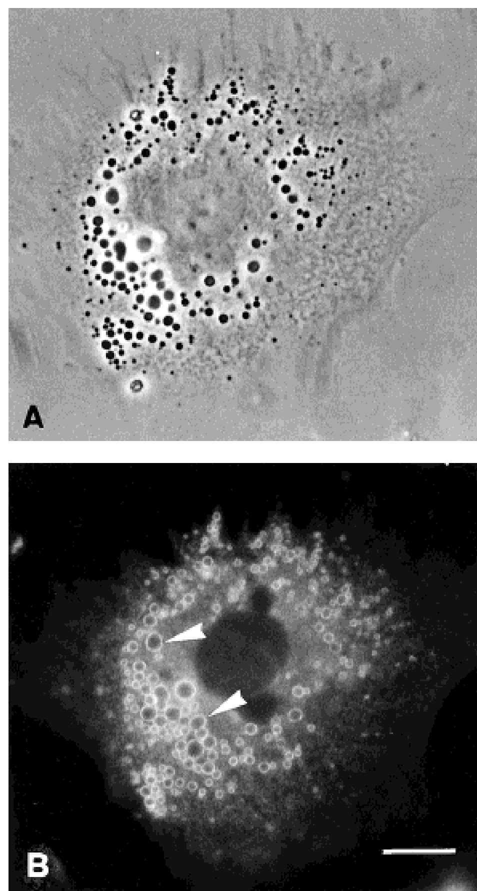

Fig. 4. Normal morphology of the lipid droplet capsule. Panel **A** is a phase pair of panel **B** (immunostained with A2 antibody). Control adrenocortical cells contain many lipid droplets surrounded by a complete bright rim (arrowheads). Scale bar = 10 μm.

DISCUSSION

This study demonstrated a direct, stimulatory effect of CS on steroid hormonal production by rat adrenal cells. The water extract of CS has been used to inhibit rejection during heart and skin transplantation in the mouse model [Zhang and Xia, 1990]. Our data suggest that the CS-induced increase in corticosterone production may be responsible for the inhibition of

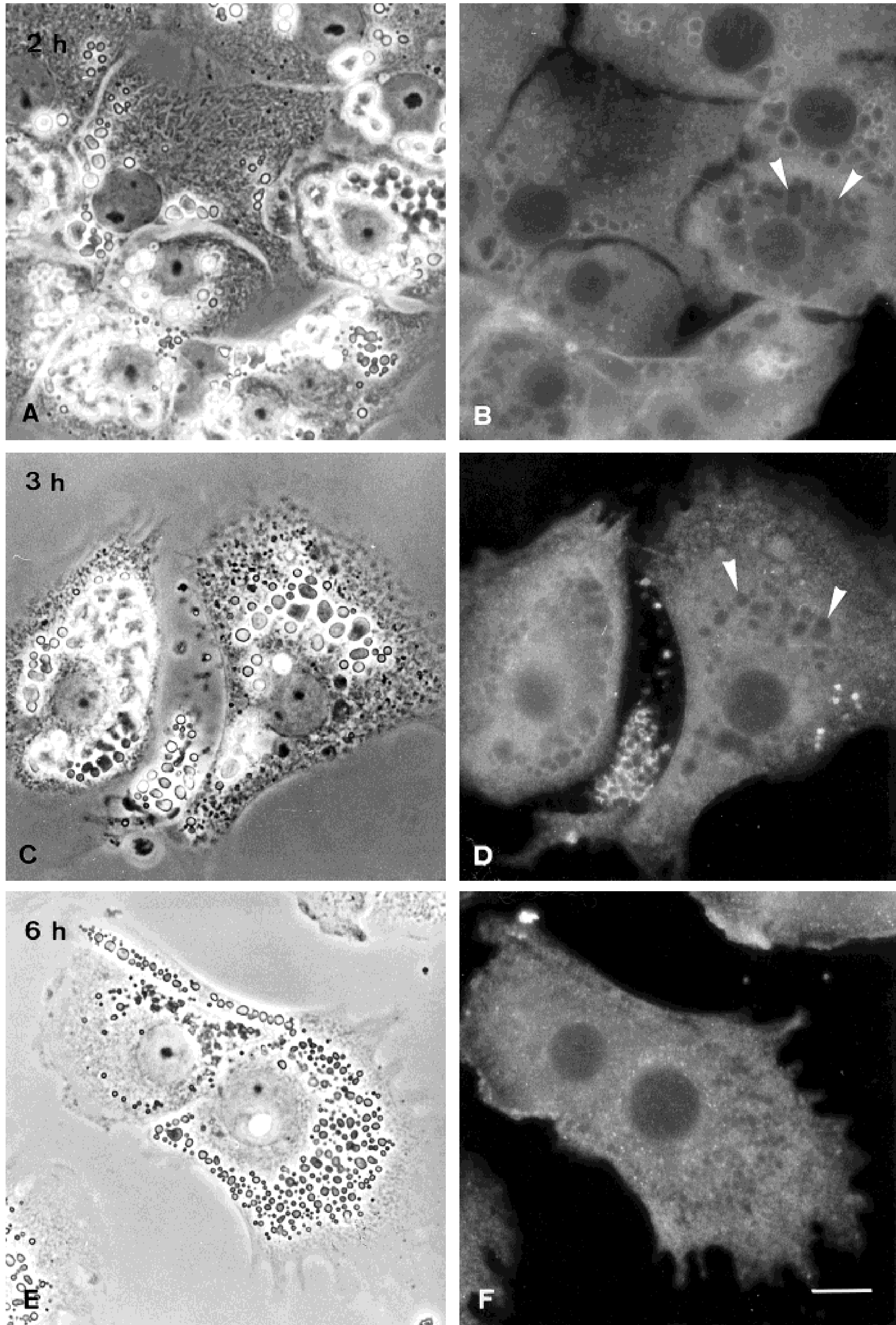

Fig. 5. Effects of CS extract on capsular morphology of lipid droplets. Panels A,C,E (immunostained using A2 antibody) are the phase pair of panels B,D,F, respectively. **A,B:** Two hours after CS (25 µg/ml) treatment. The lipid droplet capsules in some cells are incomplete or lost (arrowheads), with an increase in cytosolic stain intensity. **C,D:** Three hours after CS treatment. Most of the capsules are detached from the lipid droplet surface (arrowheads). **E,F:** Six hours after CS treatment. No staining of lipid droplet is seen by A2 antibody. Scale bar = 10 µm.

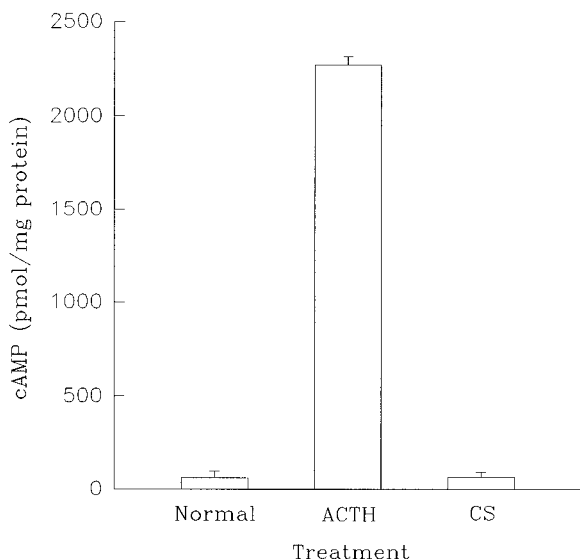

Fig. 6. Effect of CS and ACTH treatments on intracellular cAMP levels. The intracellular cAMP levels in the CS-treated group are the same as in normal cells, while ACTH induces a significant increase. Values are the mean ± SD, n = 3.

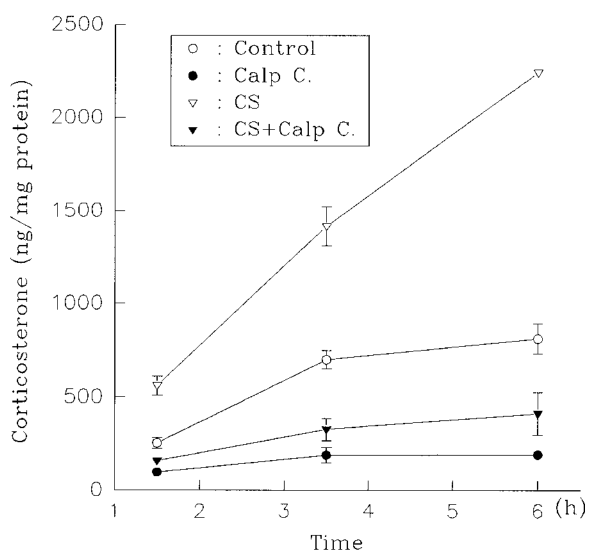

Fig. 7. Time-course study of the effect of calphostin C on CS-induced steroidogenesis. In the presence of 500 nM calphostin C, the stimulatory effect of CS (25 μg/ml) on corticosterone production is blocked 1.5 h after the treatment. (mean ± SD, n = 3).

the immune system, since glucocorticoids are able to inhibit allergic, inflammatory, and rejectory responses by regulating the expression of specific genes [Didonato et al., 1996; Parrillo and Fauci, 1979; Schleimer, 1993]. In addition, glucocorticoids accelerate apoptosis of T cells [Wyllie, 1980], which are responsible for cell-mediated immunity.

Lipid droplets in steroidogenic cells are surrounded by a proteinaceous capsule [Wang and Fong, 1995; Fong et al., 1996]. In contrast to the situation in adipocytes [Franke et al., 1987], in adrenal cells vimentin filaments do not encircle the lipid droplets in a "vimentin cage," and the capsule may therefore play a role in protecting the lipid droplet from enzyme digestion [Fong and Wang, 1997]. ACTH and dibutyryl cAMP, two PKA activators, induce decapsulation of lipid droplets and accelerate steroidogenesis in rat adrenal cells [Wang and Fong, 1995]. In Leydig cells, luteinizing hormone or dibutyryl cAMP also causes lipid droplet decapsulation [Fong et al., 1996]. The decapsulation seen in the present study correlated well with the increased steroidogenesis following CS treatment. In the present study, at a high concentration of CS, toxicity was seen, the cells retracting and occasionally becoming detatched from the substratum.

The cAMP assay indicated that the effect of CS was not mediated by the cAMP-PKA system, and we therefore propose that the effect may be mediated by the PKC system since the amounts of steroid production induced by PMA (PKC activator) and by CS are comparable [our unpublished data], and, secondly, calphostin C abolished the effect of CS on steroidogenesis. Calcium influx has been reported to activate the PKC system [Vilgrain et al., 1984; Widmaier and Hall, 1985], and it would therefore be interesting to determine whether CS influences intracellular calcium levels.

Extract of CS contains mixed products, including cordycepsin (a nucleoside derivative), polysaccharides, fatty acid compositions, adenosine, and several pyranosides [Cuningham et al., 1951; Shiao et al., 1989]. Further study is needed to characterize the functional component that stimulates steroidogenesis in adrenal cells. It is not clear whether the active component of CS binds to the membrane receptor or passively diffuses into the cells to act on steroidogenesis-related enzymes. Microinjection of CS into adrenal cells and examination of the capsular morphology of lipid droplets may provide further information.

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