[CANCER RESEARCH 44, 885–892, March 1984]

Phorbol Ester Inhibition of Ovarian and Testicular Steroidogenesis *in Vitro*[1]

Thomas H. Welsh, Jr.[2], Phillip B. C. Jones,[3] and Aaron J. W. Hsueh[4]

Department of Reproductive Medicine, M-025, University of California, San Diego, La Jolla, California 92093

ABSTRACT

Possible influences of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) upon gonadal steroidogenesis were investigated *in vitro*. Granulosa cells from hypophysectomized, estrogen-treated rats were cultured for 2 days in medium containing 0.1 μM androstenedione. Follicle-stimulating hormone (FSH) treatment increased estrogen, progesterone, and 20α-hydroxypregn-4-en-3-one production. Concomitant TPA treatment inhibited FSH-stimulated estrogen production by up to 95% [concentration that induced 50% inhibition of steroid production (ED_{50}), 1.1 ng/ml]. TPA also inhibited FSH-stimulated progesterone (ED_{50}, ~0.6 ng/ml) and 20α-hydroxypregn-4-en-3-one (ED_{50}, ~1.1 ng/ml) production. $N^6O^{2'}$-dibutyryl cyclic adenosine 3′:5′-monophosphate increased steroidogenesis; however, cotreatment with TPA blocked progestin but not estrogen production. The TPA inhibition of progestin biosynthesis was accompanied by decreases in FSH-stimulated pregnenolone biosynthesis and 3β-hydroxysteroid dehydrogenase activity without decreasing the activity of 20α-hydroxysteroid dehydrogenase. In primary cultures of rat testicular cells, human chorionic gonadotropin treatment increased testosterone production 44-fold, whereas concomitant treatment with TPA inhibited testosterone production by up to 86% (ED_{50}, 10 ng/ml). Cholera toxin and $N^6O^{2'}$-dibutyryl cyclic adenosine 3′:5′-monophosphate also increased testosterone production, while the actions of these agents were decreased by TPA. The TPA suppression of testosterone production was associated with a decrease in accumulation of 17α-hydroxyprogesterone and androstenedione and an increase in progesterone production, suggesting a specific inhibition of 17α-hydroxylase and 17,20-lyase activities. These results demonstrate the inhibitory effects of a tumor promoter upon gonadotropin-stimulated steroidogenesis by cultured rat granulosa and Leydig cells through specific regulation of steroidogenic enzymes. Additional studies may assist in further elucidation of cellular mechanisms associated with carcinogenesis and steroidogenesis.

INTRODUCTION

Phorbol esters are tetracyclic diterpenes originally identified in the seed oil of the croton plant (16, 50). These compounds promote tumor induction by carcinogenic agents. TPA,[5] a potent tumor promoter, has been utilized to study cellular mechanisms associated with carcinogenesis (5, 6, 17). Blumberg (6), Jaken *et al.* (17), and Osborne and Tashjian (41) demonstrated that rat pituitary tumor cells possess specific binding sites for phorbol esters and that TPA treatment increases prolactin and decreases growth hormone secretion by these cells *in vitro*. Additionally, TPA has been reported to increase LH and adrenocorticotropic hormone secretion by cultured rat pituitary cells (38, 48) and to stimulate pancreatic secretion of insulin (36, 51). In the present study, we have investigated the possibility that phorbol esters may affect gonadal steroidogenesis *in vitro*. Specifically, we evaluated the influence of TPA upon gonadotropin-stimulated steroidogenesis in primary cultures of rat ovarian granulosa and testicular Leydig cells.

MATERIALS AND METHODS

Reagents and Hormones. Ovine FSH (National Institutes of Health, NIH-FSH-S13; FSH activity, 15 × NIH-FSH-S1 units/mg; LH activity, 0.05 × NIH-LH-S1 units/mg; prolactin activity, <0.1% by weight) was a gift from the National Hormone and Pituitary Program, National Institute of Arthritis, Metabolism, and Digestive Diseases. HCG (CR-121; 13,450 IU/mg) was provided by the Center for Population Research, National Institute of Child Health and Human Development, NIH. Cholera toxin, $(Bu)_2$cAMP, 17β-estradiol, progesterone, 20α-OH-P, pregnenolone, Δ⁴-androstene-3,17-dione (androstenedione), 17α-hydroxyprogesterone, testosterone, bovine serum albumin (Fraction V), HEPES, TPA, and 4α-PDD were purchased from Sigma Chemical Co. (St. Louis, MO). Diethylstilbestrol was obtained from Steraloids, Inc. (Wilton, NH). Spironolactone and cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxy-androst-5-en-3-one) were provided by Dr. P. Hornsby (University of California, San Diego, CA) and J. A. Campbell (The Upjohn Co., Kalamazoo, MI), respectively.

McCoy's Medium 5A (modified, without serum), penicillin-streptomycin solution (10,000 units of penicillin base/ml and 10,000 μg of streptomycin base/ml), L-glutamine, and trypan blue were obtained from Grand Island Biological Co. (Grand Island, NY). Collagenase (144 units/mg) was purchased from Worthington Biochemical Corp. (Freehold, NJ).

[7-^{3}H]Pregnenolone (19.3 Ci/mmol), 20α-[1,2-^{3}H]hydroxyprogesterone (55.7 Ci/mmol), and [4-^{14}C]progesterone (51.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Labeled steroids were purified before use by thin-layer chromatography using the system chloroform:ether (5:1, v/v). Precoated silica plates were obtained from MC/B Manufacturing Chemists, Inc. (Cincinnati, OH).

Animals. Immature female Sprague-Dawley rats (21 to 23 days old) were hypophysectomized by Johnson Laboratories (Bridgeview, IL), and Silastic capsules (10 mm) containing diethylstilbestrol were implanted at the time of surgery. Adult male Sprague-Dawley rats (50 to 60 days of age) were also hypophysectomized by Johnson Laboratories. The ani-

Received June 17, 1983; accepted November 11, 1983.

[1] This work was supported by NIH Research Grant HD-15667 and Program Project Grant HD-12303.

[2] Recipient of a Giannini Medical Research Foundation Postdoctoral Fellowship. Present address: Physiology of Reproduction Section, Department of Animal Science, Kleberg Center, Texas A & M University, College Station, TX 77843. To whom requests for reprints should be addressed.

[3] In partial fulfillment of Ph.D. requirements. Present address: Department of Pharmacology, Stanford University, School of Medicine, Stanford, CA 94305.

[4] Recipient of Research Career Development Award HD-00375.

[5] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; FSH, follicle-stimulating hormone; 20α-OH-P, 20α-hydroxypregn-4-en-3-one; 4α-PDD, 4α-phorbol-12,13-didecanoate; $(Bu)_2$cAMP, $N^6,O^{2'}$-dibutyryl cyclic adenosine 3′:5′-monophosphate; LH, luteinizing hormone; HCG, human chorionic gonadotropin; 20α-HSD, 20α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; RIA, radioimmunoassay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; ED_{50}, concentration that induced 50% inhibition of steroid production; cAMP, cyclic AMP.

mals were given a mixture of bread, milk, dog food, water, and 0.9% NaCl solution (saline) *ad libitum* and were maintained under a 14-hr-10-hr light-dark regimen.

Steroid Production by Primary Cultures of Rat Granulosa Cells. Four to 6 days after surgery, granulosa cells were obtained from rat ovaries as detailed previously (22). These cells were cultured in 35- x 10-mm Falcon tissue culture dishes (1.5 to 2.5 × 10^5 viable cells/dish) in 1 ml of McCoy's Medium 5A supplemented with 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 100 nM androstenedione.

FSH treatment of cultured granulosa cells increases production of cAMP, pregnenolone, progesterone, and the progesterone metabolite 20α-OH-P. Granulosa cells lack the enzymes necessary to convert progesterone to androgens; therefore, the culture medium is supplemented with the aromatizable androgen androstenedione as substrate for the aromatase complexes. Under the *in vitro* conditions, FSH treatment stimulates the conversion of androstenedione to estrogen, while androstenedione also synergizes with FSH in stimulating progestin production (2, 18, 44).

To test the effect of TPA and 4α-PDD on FSH-stimulated estrogen and progestin production, granulosa cells were cultured for 2 days at 37° in a humidified 95% air:5% CO_2 incubator with appropriate test compounds. At the conclusion of the incubation period, media were collected and stored at −20° until analyzed for estrogen, progesterone, and 20α-OH-P content by RIA. To examine pregnenolone production, granulosa cells were incubated for 2 days with appropriate combinations of test compounds. Cells received fresh media containing 1 μM cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase (15), and were incubated for 25 min. After the 25-min incubation period, the appropriate amounts of TPA and FSH were added back to the cultures, and the cells were reincubated for 4 hr. Pregnenolone accumulation during the 4-hr incubation was measured by RIA.

Steroid Production by Primary Cultures of Rat Testicular Cells. Testes of adult hypophysectomized rats were excised 12 to 15 days after hypophysectomy. Testicular cell suspensions were prepared as described previously (21, 53) by incubating decapsulated testes at 37° for 1.5 hr in an enzyme solution containing 0.4% collagenase, DNase (10 μg/ml), and 0.1% bovine serum albumin in HEPES buffer (137 nM NaCl:5 mM KCl:0.7 mM $NaHPO_4$:25 mM HEPES:10 mM glucose:360 μM $CaCl_2$). Cells were washed and resuspended in culture medium, and cell viability (90 to 95%) was quantitated by trypan blue exclusion. Approximately 0.9 to 1.1 × 10^6 cells/culture were maintained at 37° under 95% air:5% CO_2 in McCoy's Medium 5A supplemented with penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and L-glutamine (2 mM). Media were changed every 2 days for 8 days. Testicular cells were treated with appropriate combinations of TPA, 4α-PDD, and hormones between Days 8 and 10 of culture, and media were collected for steroid RIA.

RIAs. Medium concentrations of progesterone and estrogen were determined by RIA with specific antisera provided by Dr. G. Abraham as described previously (52). Medium concentrations of 20α-OH-P were determined by RIA (31) using specific antisera supplied by Ralph Schwall (University of California, San Diego, CA). Medium concentration of pregnenolone was measured with a specific antiserum (Pantex, Santa Monica, CA) (28). Medium concentrations of testosterone and androstenedione were determined by RIA (3) with specific antisera (<0.01% cross-reaction with progesterone and 17α-hydroxyprogesterone) provided by Dr. G. Abraham. In some experiments, intermediates of the testosterone biosynthetic pathway were separated by Celite column chromatography and measured by specific RIA (1).

Enzyme Assays. The assay used to measure 20α-HSD (EC 1.1.1.149) activity was based on a procedure developed by Eckstein *et al.* (12) and modified by us, in which 20α-HSD activity was measured as the rate of conversion of [^{3}H]-20α-OH-P to [^{3}H]progesterone (29). The assay of 3β-HSD/$\Delta^{5,4}$-isomerase (EC 1.1.1.51/EC 5.3.3.1) activity was based on a procedure developed by Murono and Payne (37) in which 3β-HSD activity was measured as the rate of conversion of pregnenolone to progesterone. Our modification of this procedure has been described elsewhere (30). The Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) was utilized to determine cellular protein content (7). Bovine γ-globulin was used as the standard for the protein assay.

Data Analysis. RIA data were analyzed with a program which utilizes a weighted logit-log regression analysis (11). A 4-parameter logistic curve-fitting program was used to obtain dose-response curves (26). Experimental data are presented as the means of measurements of quadruplicate cultures. Comparable results were obtained in 3 to 5 replicates of each experiment. Treatment differences were tested by analysis of variance, Dunnet's test, and Student's *t* test (49). Comparisons with $p > 0.05$ were not considered significant.

RESULTS

Influence of TPA and 4α-PDD on FSH-stimulated Steroidogenesis in Granulosa Cells. Granulosa cells were cultured with medium alone (control), TPA (100 ng/ml), 4α-PDD (100 ng/ml), or FSH in the presence or absence of increasing concentrations of TPA or 4α-PDD (Chart 1). Negligible levels of estrogen were produced by control cultures and cultures treated with either TPA or 4α-PDD alone (Chart 1*A*). Administration of FSH increased estrogen production by 68-fold relative to controls. However, concomitant administration of TPA inhibited the FSH action in a dose-dependent manner (ED_{50}, ~1.1 ng/ml), with maximal inhibition (95%) achieved at 10 ng/ml. In contrast, addition of 4α-PDD (100 ng/ml) did not affect FSH-stimulated estrogen production.

With regard to progesterone production, treatment with either TPA or 4α-PDD did not alter basal production (Chart 1*B*). Addition of FSH stimulated progesterone production by 80-fold, whereas concurrent addition of TPA but not 4α-PDD suppressed the FSH action (ED_{50}, ~0.6 ng/ml). As shown in Chart 1*C*, control and 4α-PDD-treated cultures produced negligible levels of 20α-OH-P (<100 pg/ml), whereas treatment with TPA alone increased 20α-OH-P levels to ~1 ng/ml. Furthermore, addition of FSH increased 20α-OH-P production by 75-fold, while concomitant treatment with TPA inhibited FSH-stimulated 20α-OH-P production, with an ED_{50} of ~1.1 ng/ml. The stimulatory effect of FSH on 20α-OH-P production was not affected by treatment with 4α-PDD.

Whether the inhibitory actions of TPA on FSH-stimulated steroid production occur at higher concentrations of FSH was also investigated. Granulosa cells were cultured in medium alone, with TPA (100 ng/ml) or with increasing concentrations of FSH (0.1 to 100 ng/ml) in the presence or absence of TPA. FSH treatment increased estrogen, progesterone, and 20α-OH-P production in a dose-dependent manner. Concomitant TPA treatment inhibited ($p < 0.05$) these stimulatory actions of FSH (data not shown).

Effect of TPA on $(Bu)_2$cAMP-stimulated Steroid Production. The stimulatory effects of FSH on granulosa cell steroidogenesis are presumed to be mediated via increased cAMP production. Therefore, the influence of TPA on $(Bu)_2$cAMP action was examined (Chart 2). Granulosa cells were cultured in medium alone (control) or with $(Bu)_2$cAMP, in the absence or presence of TPA (1, 10, or 100 ng/ml). Relative to controls, treatment with $(Bu)_2$cAMP increased production of estrogen, progesterone, and 20α-OH-P by 31-, 40-, and 80-fold, respectively. The stimulation of estrogen production by $(Bu)_2$cAMP was not affected ($p > 0.05$) by TPA treatment. In contrast, $(Bu)_2$cAMP-stimulated production of progesterone and 20α-OH-P was inhibited by TPA in a dose-related manner. It appears that at least part of the

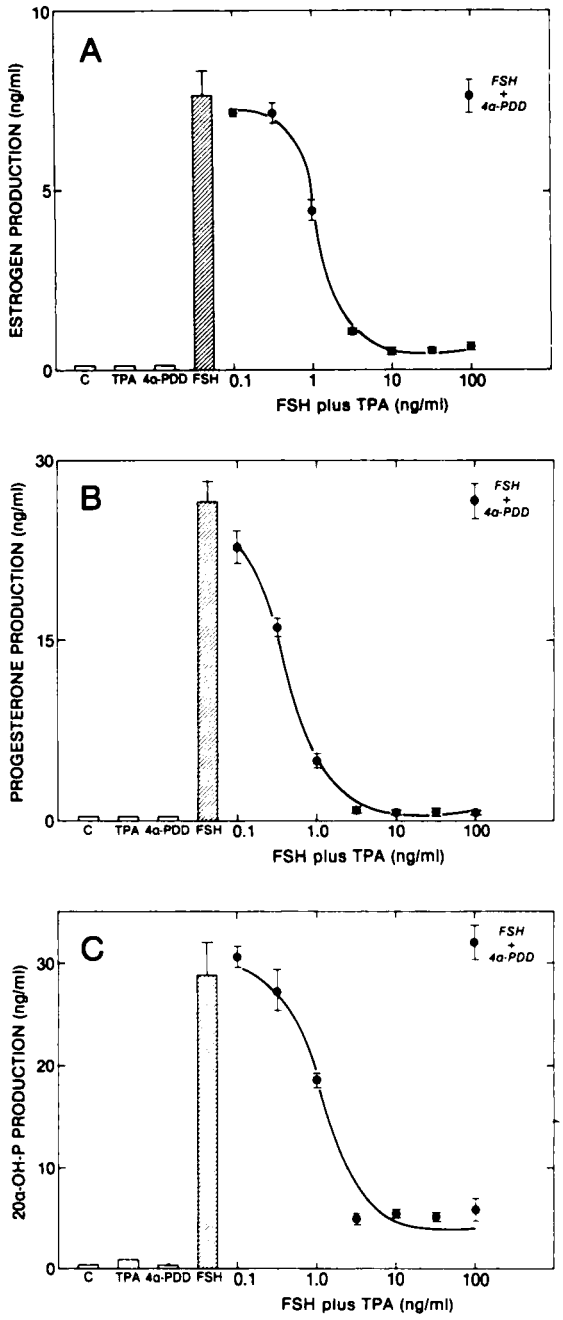

Chart 1. Dose-dependent effect of TPA and 4α-PDD on FSH-stimulated production of estrogen, progesterone, and 20α-OH-P by cultured granulosa cells. Granulosa cells obtained from immature hypophysectomized rats were cultured for 48 hr in medium alone [control (*C*)], with TPA (100 ng/ml) or 4α-PDD (100 ng/ml), or with FSH (10 ng/ml) (▨) in the absence or presence of increasing concentrations of TPA or 4α-PDD (100 ng/ml). Medium concentrations of estrogen (*A*), progesterone (*B*), and 20α-OH-P (*C*) were measured by RIA. Each *point* represents the mean of quadruplicate cultures. *Bars*, S.E.

inhibitory effect of TPA on FSH-stimulated progestin production occurs distal to the formation of cAMP.

Effect of TPA and FSH on 3β-HSD and 20α-HSD Activities of Granulosa Cells. The influence of TPA treatment on the activities of 3β-HSD and 20α-HSD was examined by culturing granulosa cells for 2 days in medium alone (control), with TPA (5 ng/ml), with FSH (10 ng/ml), or with FSH plus TPA (Chart 3). Pregnenolone is converted to progesterone by the enzyme 3β-HSD, whereas 20α-HSD converts progesterone to the less active progestin metabolite 20α-OH-P.

Relative to control cultures, treatment with FSH or TPA increased 3β-HSD activity 2.9- and 1.9-fold, respectively. Concomitant TPA treatment inhibited FSH-stimulated 3β-HSD activity by 20%. With regard to 20α-HSD activity, treatment with TPA alone increased enzyme activity by 6.8-fold, but FSH treatment did not affect 20α-HSD activity. Treatment with FSH plus TPA resulted in a higher level of 20α-HSD activity than that obtained with FSH alone (a 6.1-fold increase).

Effect of TPA on FSH-stimulated Production of Pregnenolone. Whether TPA affects the conversion of cholesterol to pregnenolone was evaluated. Granulosa cells were cultured for 2 days in medium alone (control) or with TPA (50 ng/ml), FSH (10 ng/ml), or FSH plus TPA. After 2 days, cells were treated with cyanoketone (to inhibit 3β-HSD activity) and various test compounds as described in "Materials and Methods." Control cultures produced pregnenolone (346 pg/ml), whereas treatment with TPA alone increased ($p < 0.05$) pregnenolone production

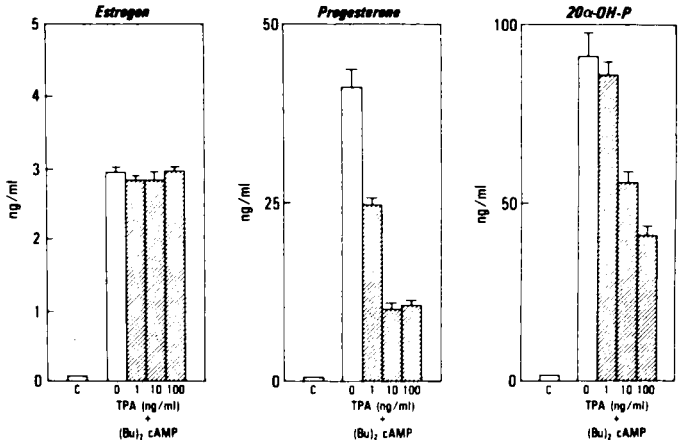

Chart 2. Effect of TPA on $(Bu)_2$cAMP-stimulated production of estrogen, progesterone, and 20α-OH-P by cultured granulosa cells. Granulosa cells were cultured for 48 hr in medium alone [control (*C*)], with TPA (100 ng/ml), or with $(Bu)_2$cAMP (5 mg/ml) in the absence (□) or presence (▨) of TPA (1, 10, or 100 ng/ml). Medium concentrations of estrogen, progesterone, and 20α-OH-P were measured by RIA. Each *point* represents the mean of quadruplicate cultures. *Bars*, S.E.

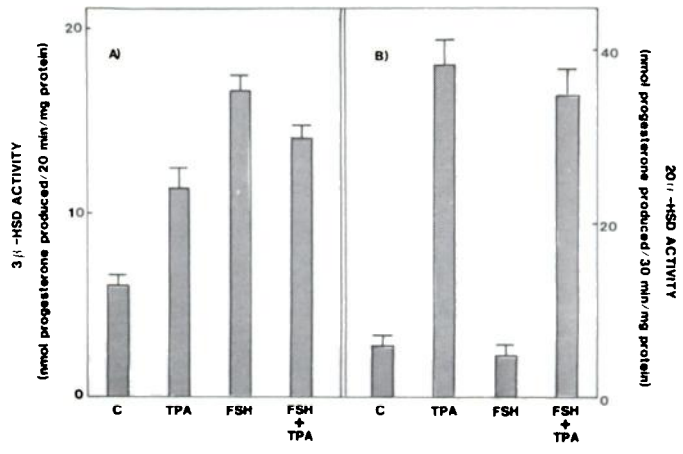

Chart 3. Effects of TPA and FSH on 3β-HSD and 20α-HSD activities of cultured granulosa cells. Granulosa cells (4×10^5 viable cells/dish) were cultured for 2 days in medium alone [control (*C*)] or with TPA (5 ng/ml), FSH (10 ng/ml), or FSH plus TPA. Enzyme activities were determined as described in "Materials and Methods." Each *point* represents the mean of 16 determinations from 4 experiments. *Bars*, S.E.

up to 690 pg/ml. FSH treatment stimulated a 42.6-fold increase in pregnenolone production, while concomitant treatment with TPA suppressed FSH-stimulated pregnenolone production by 86.7%. These observations suggest that TPA inhibits FSH-stimulated progestin production via a decrease in enzymes prior to pregnenolone formation.

Effect of TPA on HCG-stimulated Testosterone Production by Cultured Leydig Cells. Primary cultures of testicular cells were treated for 48 hr with medium alone (control), TPA (100 ng/ml) 4α-PDD (100 ng/ml), or HCG (10 ng/ml) in the absence or presence of TPA (0.1 to 100 ng/ml) or 4α-PDD (100 ng/ml) (Chart 4). Medium concentration of testosterone was negligible in control cultures and cultures treated with TPA or 4α-PDD alone. Addition of HCG increased testosterone production 39-fold relative to controls. Concurrent addition of TPA suppressed HCG-stimulated testosterone production in a dose-related manner (ED_{50}, ~10 ng/ml). At 100 ng/ml, TPA inhibited HCG action by 86%. In contrast, 4α-PDD (100 ng/ml) did not alter HCG action.

Effect of TPA on Cholera Toxin and $(Bu)_2$cAMP-stimulated Testosterone Production. Since the gonadotropins LH and HCG are presumed to stimulate testicular Leydig cell steroidogenesis via increased cAMP production, the influence of TPA upon the ability of cholera toxin (a cAMP-stimulating agent) and $(Bu)_2$cAMP (a cAMP analogue) to stimulate testosterone production was evaluated (Chart 5). Treatment with cholera toxin increased testosterone production 23-fold relative to controls. Concomitant addition of TPA (10 and 100 ng/ml) suppressed cholera toxin-stimulated testosterone production 78 and 93%, respectively. Similarly, $(Bu)_2$cAMP treatment resulted in a 38-fold increase in testosterone production, while the stimulatory effect of the cAMP analogue was suppressed 64 and 87% by 10 and 100 ng of TPA, respectively, per ml. Thus, part of the inhibitory effect of TPA on HCG-stimulated testosterone production may occur distal to cAMP formation.

Effect of TPA on HCG-stimulated Production of Intermediate Steroids of the Testosterone Biosynthetic Pathway.

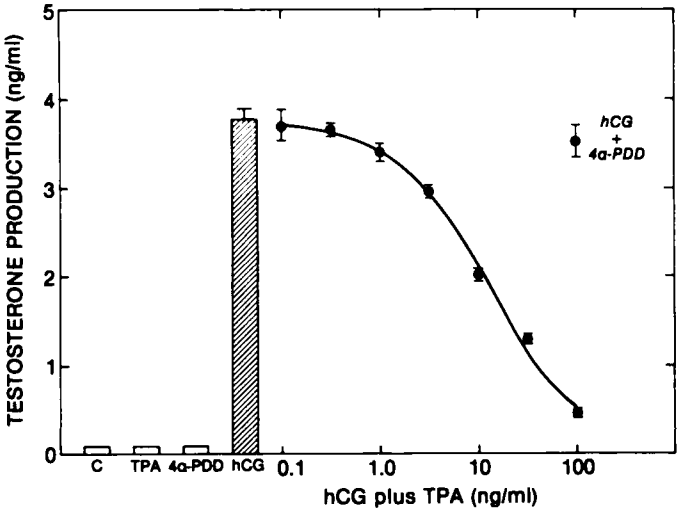

Chart 4. Effect of TPA and 4α-PDD on HCG-stimulated testosterone production by cultured testicular cells. Primary cultures of testicular cells were prepared as described in "Materials and Methods." On Day 8, cultures were treated with medium alone [control (*C*)], with TPA (100 ng/ml) or 4αPDD (100 ng/ml), or with HCG (10 ng/ml) in the absence (■) or presence of increasing concentrations of TPA or 4α-PDD (100 ng/ml). Media were collected on Day 10 of culture, and the concentration of testosterone was measured by RIA. Each *point* represents the mean of quadruplicate cultures. *Bars*, S.E.

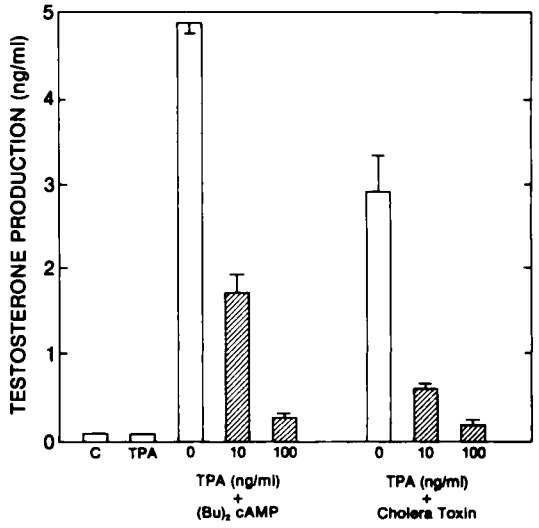

Chart 5. Effect of TPA treatment on testosterone production induced by $(Bu)_2$cAMP and cholera toxin in cultured testicular cells. Primary cultures of testicular cells were maintained as described in "Materials and Methods." On Day 8, cultures were treated with medium alone [control (*C*)], with TPA (100 ng/ml), or with cholera toxin (10 ng/ml) or $(Bu)_2$cAMP) (0.5 mg/ml) in the absence (□) or presence (▨) of TPA (10 or 100 ng/ml). Media were collected on Day 10 of culture, and the concentration of testosterone was determined by RIA. Each *point* represents the mean of quadruplicate cultures. *Bars*, S.E.

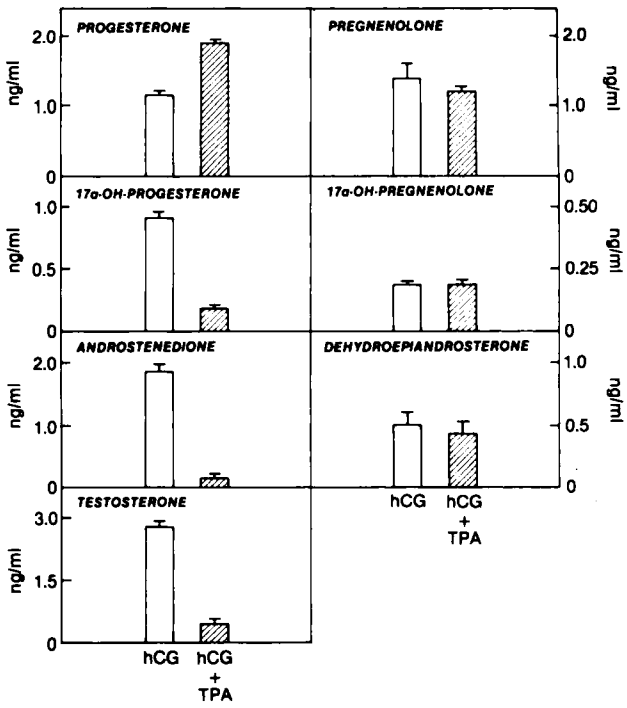

Chart 6. Effect of TPA treatment on HCG-stimulated production of intermediate steroids of the testosterone biosynthetic pathway. Testicular cell cultures were treated with medium containing either HCG (10 ng/ml) (□) or HCG plus TPA (100 ng/ml) (▨) for 2 days. Media were collected, and intermediate steroids of the testosterone biosynthetic pathway were separated by celite column chromatography for RIA. Each *point* represents the mean of quadruplicate cultures. *Bars*, S.E.

Whether TPA treatment influences steroidogenic enzymes was investigated by measurement of the medium concentrations of various intermediate steroids of the testosterone-biosynthetic pathway in cells treated with HCG or HCG plus TPA (Chart 6).

Mammalian Leydig cells can form testosterone via 2 main pathways identified as (*a*) Δ^4 pathway; pregnenolone → proges-

terone → 17α-hydroxyprogesterone → androstenedione → testosterone; and (*b*) the Δ^5 pathway; pregnenolone → 17α-hydroxypregnenolone → dehydroepiandrosterone → androstenedione → testosterone. In the rat Leydig cells, the Δ^4 pathway appeared to be the preferred pathway for testosterone synthesis, as the concentration of Δ^4 steroids was greater than that of Δ^5 steroids. As observed previously, TPA treatment suppressed HCG-stimulated testosterone production by 82%. Similarly, TPA decreased HCG-stimulated production of androstenedione and 17α-hydroxyprogesterone by 88 and 80%, respectively. In contrast, HCG-stimulated production of progesterone was increased 58% by TPA treatment, while HCG-stimulated pregnenolone production was unaffected by TPA. These results suggest a possible inhibitory action of TPA upon the 17α-hydroxylase enzyme which converts progesterone to 17α-hydroxyprogesterone.

Influence of TPA on the Conversion of Exogenous Progesterone and 17α-Hydroxyprogesterone to Androgens. The possibility that TPA may decrease the activity of the steroidogenic enzymes 17α-hydroxylase and 17,20-lyase was further examined by measurement of androgen production subsequent to the addition of exogenous substrates to testicular cells treated with HCG and/or TPA (Charts 7 and 8). Testosterone production by control, TPA-treated, and 4α-PDD-treated cultures was low (<100 pg/ml). Addition of exogenous progesterone increased the medium concentration of testosterone to ~780 pg/ml for both control and 4α-PDD-treated cultures, but only to 470 pg/ml for TPA-treated cultures (Chart 7). Addition of HCG increased testosterone production 43.8-fold relative to controls, while concomitant addition of progesterone further increased testosterone production by 5.9-fold. However, concurrent treatment with TPA suppressed HCG-stimulated testosterone production in either the absence (an 89% decrease) or presence (a 98% decrease) of exogenous progesterone. In contrast, cotreatment with 4α-PDD did not affect HCG-stimulated testosterone production for cultures treated with or without progesterone. These data indicate that TPA may decrease 17α-hydroxylase and/or 17,20-lyase activity.

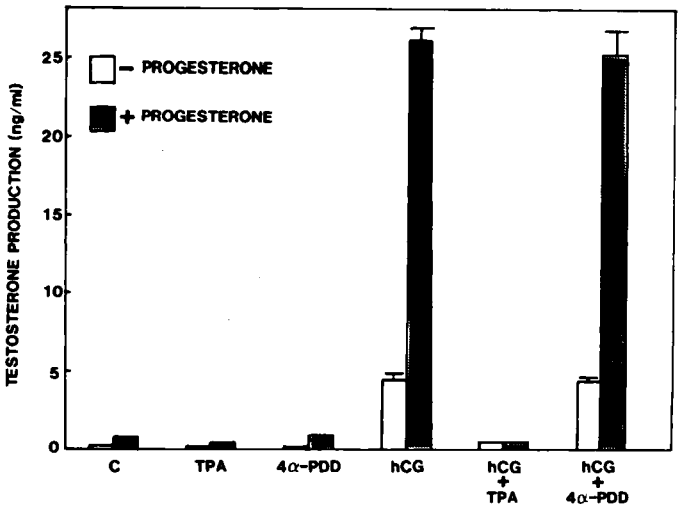

Chart 7. Effect of TPA treatment on the conversion of exogenous progesterone to testosterone by cultured testicular cells. Testicular cell cultures were treated with or without HCG (10 ng/ml) and/or TPA or 4α-PDD (10 ng/ml). Some cells from each group were also treated with 10 μM progesterone (▨). Media were collected 48 hr later for testosterone RIA. Each *point* represents the mean of quadruplicate cultures. *Bars*, S.E.

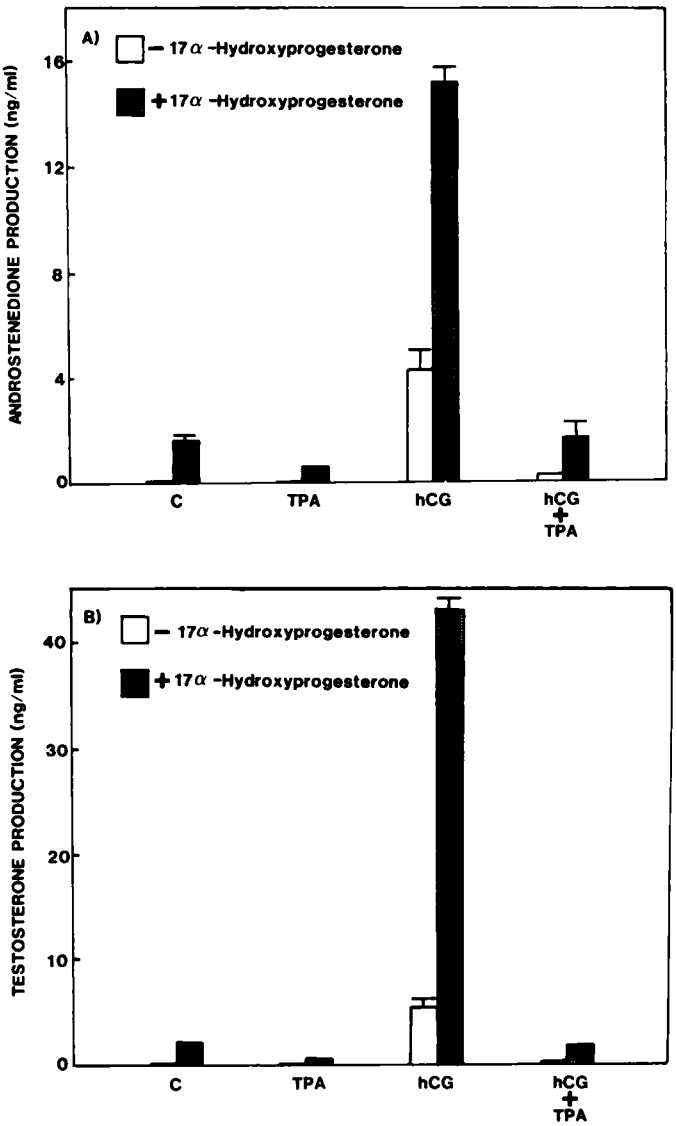

Chart 8. Effect of TPA treatment on the conversion of exogenous 17α-hydroxyprogesterone to androstenedione and testosterone by cultured testicular cells. Testicular cell cultures were treated with HCG (10 ng/ml) and/or TPA (100 ng/ml). Some cell cultures from each group were also treated with 10 μM 17α-hydroxyprogesterone (▨). Media were collected 48 hr later, and the concentrations of androstenedione (*A*) and testosterone (*B*) were determined by RIA. Each *point* represents the mean of quadruplicate cultures. *Bars*, S.E.

The possible influence of TPA on the activity of 17,20-lyase was tested by measurement of medium concentrations of androstenedione and testosterone in cultures treated with exogenous 17α-hydroxyprogesterone and appropriate combinations of test compounds (Chart 8). In the absence of exogenous 17α-hydroxyprogesterone, control and TPA-treated cultures produced low levels of androstenedione and testosterone. Addition of 17α-hydroxyprogesterone to control cultures increased ($p < 0.05$) androstenedione and testosterone production. In contrast, TPA treatment reduced the conversion of exogenous 17α-hydroxyprogesterone to androstenedione (a 60% decrease) and testosterone (a 78% decrease).

Treatment with HCG increased medium concentrations of androstenedione and testosterone 43.1- and 54.8-fold, respectively. When cells were treated with both HCG and 17α-hydroxyprogesterone, the production of androstenedione and testos-

terone was further increased 3.5- and 7.9-fold, respectively. In the absence or presence of exogenous 17α-hydroxyprogesterone, concomitant treatment with TPA but not 4α-PDD (data not shown) suppressed by approximately 90% the HCG-stimulated production of androstenedione and testosterone. Therefore, TPA may decrease the activity of 17,20-lyase.

DISCUSSION

These results demonstrate the inhibitory effects of TPA, a phorbol ester with potent tumor-promoting activity, upon gonadotropin-stimulated steroidogenesis by cultured gonadal cells. TPA treatment inhibits FSH-stimulated estrogen, progesterone, and 20α-OH-P production in cultured ovarian granulosa cells, as well as HCG-stimulated testosterone production by cultured testicular Leydig cells. The inhibitory actions of TPA appear to be specific, as the phorbol congener 4α-PDD was ineffective. Also, the TPA-induced inhibition of steroid production occurs at low concentrations (ED_{50}s between 0.5 and 16 nM). This is comparable to the affinity of TPA for specific phorbol ester binding sites found on the plasma membrane of a variety of cell types (27, 45).

TPA is one of the tetracyclic diterpene compounds known as tumor promoters (5, 6, 16, 17, 50). TPA has been reported to elicit pleiotrophic responses in a variety of cell types. TPA-stimulated epidermal proliferation in mice is associated with increased prostaglandin content, ornithine decarboxylase activity, histone phosphorylation, and phospholipid synthesis (16, 17). Phorbol esters also inhibit or induce cell differentiation in various cell types (9, 14, 23, 24, 33, 34, 54). Further, TPA treatment stimulates polyamine synthesis by human promyelocytic leukemia cells (24) and prostaglandin synthesis by MDCK kidney cells (32). However, *in vitro* studies have yet to conclusively identify the mechanism(s) whereby TPA exerts its varied effects upon cellular functions.

With regard to endocrine cell types, Jaken *et al.* (27) have identified specific binding sites for phorbol esters on cultured rat pituitary tumor cells. Phorbol ester binding has also been reported in hamster ovarian (45) and human mammary tumor cells (40). TPA treatment increases prolactin and decreases growth hormone secretion by pituitary tumor cells *in vitro* (41). Stimulatory effects of TPA upon secretion of other anterior pituitary hormones (*e.g.*, LH and adrenocorticotropic hormone) have also been reported (38, 48). Additionally, phorbol ester treatment induces HCG secretion by cultured human choriocarcinoma cells (25) and stimulates somatostatin release by cultured brain cells (43). The present data not only extend the concept that phorbol esters affect specific endocrine cells by inclusion of the granulosa and Leydig cells but also provide insight relevant to the mechanism whereby TPA inhibits gonadotropin-stimulated steroidogenesis.

In the granulosa cells, TPA inhibits the aromatase enzymes which catalyze the conversion of androgens to estrogens. With regard to progestin production, TPA appears to decrease FSH stimulation of enzymes involved in pregnenolone biosynthesis. Additionally, FSH-stimulated 3β-HSD activity was slightly reduced by TPA, whereas 20α-HSD activity was stimulated. Furthermore, the inhibitory effect of TPA on FSH-stimulated progestin production appears to be mediated in part distal to cAMP, whereas the absence of an effect of TPA upon $(Bu)_2$cAMP-stimulated estrogen production suggests that TPA suppression of FSH-induced estrogen production occurs prior to cAMP. These divergent effects of TPA indicate that FSH-stimulated estrogen and progestin production may be regulated via different pathways. Possible TPA modulation of cAMP production is presently under study.

Interestingly, pregnenolone production and the actvities of 3β-HSD and 20α-HSD were increased in cells treated with TPA alone. These observations suggest that, in the absence of gonadotropin treatment, TPA stimulates pregnenolone biosynthesis and that the associated elevations in 3β-HSD and 20α-HSD activities result in an increased medium accumulation of 20α-OH-P. TPA can now be included with epidermal growth factor and gonadotropin-releasing hormone as a substance which stimulates 20α-OH-P production in granulosa cells independent of actions exerted by gonadotropins (29, 31).

TPA suppresses testosterone production in testis cells treated with HCG or a cAMP analogue, $(Bu)_2$cAMP, indicative of a post-cAMP site of action for TPA. Whether TPA affects specific steroidogenic enzymes was examined by measurement of intermediate steroids of the testosterone-biosynthetic pathway and by monitoring the conversion of exogenous progestins to androgens in cells treated with TPA. The TPA inhibition of HCG-stimulated testosterone production was associated with greater than 80% decreases in production of androstenedione and 17α-hydroxyprogesterone. In contrast, TPA increased HCG-stimulated progesterone production, while pregnenolone production was unchanged. Further, TPA suppressed the conversion of the exogenous progestins, progesterone and 17α-hydroxyprogesterone, to androgens. Taken together, these experiments suggest an inhibitory effect of TPA upon the activities of 17α-hydroxylase and 17,20-lyase. In addition, our preliminary study indicates that, in the presence of cyanoketone and spironolactone, HCG-stimulated pregnenolone production is not affected by TPA treatment [HCG, 4.2 ± 0.1 (S.E.) ng/ml; HCG plus TPA, 4.5 ± 0.4 ng/ml], suggesting a lack of TPA effect on enzymes prior to pregnenolone biosynthesis.

The suppressive effects of TPA upon gonadal steroidogenesis are not related to a general inhibition of cellular functions by TPA. Treatment with TPA alone markedly enhanced both 3β-HSD and 20α-HSD activities and 20α-OH-P accumulation in cultured granulosa cells. Further, TPA treatment for 2 days did not affect the viability of granulosa cells (data not shown). Similarly, the inhibitory effect of TPA upon HCG-stimulated androgen biosynthesis is associated with increases in progesterone accumulation.

The mechanisms by which TPA modulates diverse steroidogenic enzymes in the gonadal cells are not known. In several cell types, phorbol esters stimulate phospholipase activity, which may, in turn, regulate plasma membrane phospholipid turnover and methylation (19). Davis *et al.* (10), Farese *et al.* (13), and Lowitt *et al.* (35) have suggested that phospholipid metabolism plays an important intermediary role in the regulation of steroidogenesis by pituitary hormones. Furthermore, TPA also decreases the binding of thyrotropin-releasing hormone and somatostatin in cultured rat pituitary cells (42) and uncouples the interaction between β-adrenergic receptors and adenyl cyclase activation in epidermal cells (4). It is unknown whether TPA inhibition of gonadal steroidogenesis observed in the present study may reflect an effect of TPA on gonadotropin binding, adenyl cyclase activation, and/or phospholipid turnover in the cultured cells.

Recent investigations suggest that regulation of the activity of protein kinase C, a membrane-associated, phospholipid-depend-

ent, and calcium-activated protein kinase, may be important in transmembrane signaling processes. TPA enhances protein kinase C-mediated phosphorylation of proteins in human platelet cells and rat brain cells (8, 39). These results raise the intriguing possibility that a membrane-bound protein kinase may be a phorbol ester receptor or part of the receptor in gonadal and other cell types (39).

The physiological or pharmacological significance of the present findings is unclear at this time. One may speculate that the multiple levels of endocrine aberrations induced by phorbol esters may be related to their tumor-promoting ability. The intriguing findings of specific, high-affinity binding sites and profound biological effects in various vertebrate cell types for a substance of plant origin have prompted the search for putative endogenous ligands or phorbol ester-like substances. The endogenous ligand has yet to be identified. However, Horowitz *et al.* (20) identified a phorbol ester binding inhibitor in amniotic fluid and serum, while Shoyab and Todaro (46) isolated a serum phorbol ester binding protein distinct from the phorbol ester receptor. Moreover, a TPA-degrading enzyme has been found in the liver of mice (47). The possible presence of phorbol ester binding sites or metabolizing enzymes of gonadal origin remains to be established.

In conclusion, the present data demonstrate that a tumor promoter of plant origin exerts significant, specific actions upon specialized endocrine functions of differentiated gonadal cell types. Studies of TPA actions in these cell models may further enhance our insight regarding mechanisms of steroid production and carcinogenesis.

ACKNOWLEDGMENTS

We thank L. Tucker, C. Valk, C. Talamantez, and B. Hopper for technical assistance and K. Watts for manuscript preparation. We also thank Dr. R. E. Canfield and the Center for Population Research of the National Institute of Child Health and Human Development for HCG, Dr. D. Rodbard (National Institute of Child Health and Human Development, NIH) for the 4-parameter logistic curve-fitting program, and the National Hormone and Pituitary Program, National Institute of Arthritis, Metabolism, and Digestive Diseases, for ovine FSH.

REFERENCES

1. Anderson, D. C., Hopper, B. R., Lasley, B. L., and Yen, S. S. C. A simple assay method for the assay of eight steroids in small volumes of plasma. Steroids, *28:* 179–195, 1976.
2. Armstrong, D. T., and Dorrington, J. H. Androgens augment FSH-induced progesterone secretion by cultured rat granulosa cells. Endocrinology, *99:* 1411–1414, 1976.
3. Bambino, T. H., Schreiber, J. R., and Hsueh, A. J. W. Gonadotropin-releasing hormone and its agonist inhibit testicular steroidogenesis in immature and adult hypophysectomized rats. Endocrinology, *107:* 908–917, 1980.
4. Belman, S., and Garte, S. Phorbol myristate acetate uncouples the relationship between β-adrenergic receptors and adenyl cyclase in mouse epidermis: a phenotypic trait in papillomas. Carcinog. Compr. Surv., *7:* 561–562, 1982.
5. Blumberg, P. M. *In vitro* studies on the mode of action of the phorbol esters, potent tumor promoters: Part I. CRC Crit. Rev. Toxicol., *8:* 153–197, 1980.
6. Blumberg, P. M. *In vitro* studies on the mode of action of the phorbol esters, potent tumor promoters: Part II. CRC Crit. Rev. Toxicol., *9:* 199–234, 1980.
7. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. Anal. Biochem., *72:* 248–254, 1976.
8. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, U. Direct action of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem., *257:* 7847–7851, 1982.
9. Cohen, R., Pacifici, M., Rubinstein, N., Biehl, J., and Holtzer, H. Effect of a tumour promoter on myogenesis. Nature (Lond.), *266:* 538–540, 1977.
10. Davis, J. S., Farese, R. V., and Marsh, J. M. Stimulation of phospholipid labeling and steroidogenesis by luteinizing hormone in isolated bovine luteal cells. Endocrinology, *109:* 469–475, 1981.
11. Davis, S. E., Jaffe, M. L., Munson, P. J., and Rodbard, D. Radioimmunoassay Data Processing With a Small Programmable Calculator (Technical Report). Bethesda, MD: NIH, 1979.
12. Eckstein, B., Raanan, M., Lerner, N. Cohen, S., and Nimrod, A. The appearance of 20α-hydroxysteroid dehydrogenase activity in preovulatory follicles of immature rats treated with pregnant mare serum gonadotropin. J. Steroid Biochem., *8:* 213–216, 1977.
13. Farese, R. V., Sabir, A. M., and Vandor, S. L. Adrenocorticotropin acutely increases adrenal polyphosphoinositides. J. Biol. Chem., *254:* 6842–6848, 1979.
14. Glimelius. B., and Oneston, J. A. Analysis of developmentally homogenous neural crest cell populations *in vitro*. II. A tumor-promoter (TPA) delays differentiation and promotes cell proliferation. Dev. Biol., *82:* 95–101, 1981.
15. Goldman, A. S., Yakovac, W. C., and Bongiovanni, A. M. Persistent effects of synthetic androstene derivative on activities of 3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase in rats. Endocrinology, *77:* 1105–1118, 1965.
16. Hecker, E. Cocarcinogenic principles from the seed oil of *Croton tiglium* and from other Euphorbiaceae. Cancer Res., *28:* 2338–2348, 1968.
17. Hecker, E., N. E. Fusenig, W. Kunz, F. Marks, and H. W. Thielmann (eds.), Carcinogenesis—A Comprehensive Survey, Vol. 7: Cocarcinogenesis and Biological Effects of Tumor Promoters. New York: Raven Press, 1982.
18. Hillier, S. G., Zeleznik, A. J., and Ross, G. T. Independence of steroidogenic capacity and luteinizing hormone receptor induction in developing granulosa cells. Endocrinology, *102:* 937–946, 1978.
19. Hirata, F., and Axelrod, J. Phospholipid methylation and biological signal transmission. Science (Wash. D. C.), *209:* 1082–1090, 1980.
20. Horowitz, A. D., Greenbaum, E., and Weinstein, I. B. Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biologic fluids. Proc. Natl. Acad. Sci. U. S. A., *78:* 2315–2319, 1981.
21. Hsueh, A. J. W. Gonadotropin stimulation of testosterone production in primary cultures of adult rat testis cells. Biochem. Biophys. Res. Commun., *97:* 506–512, 1980.
22. Hsueh, A. J. W., Wang, C., and Erickson, G. F. Direct inhibitory effect of gonadotropin-releasing hormone upon follicle-stimulating hormone induction of luteinizing hormone receptor and aromatase activity in rat granulosa cells. Endocrinology, *106:* 1697–1705, 1980.
23. Huberman, E., and Callaham, M. F. Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. Proc. Natl. Acad. Sci. U. S. A., *76:* 1293–1297, 1979.
24. Huberman, E., Welks, C., Herrmann, P., Callaham, M., and Slaga, T. Alterations in polyamine levels induced by phorbol diesters and other agents that promote differentiation in human promyelocytic leukemia cells. Proc. Natl. Acad. Sci. U. S. A., *78:* 1062–1066, 1981.
25. Ilekis, J., and Benveniste, R. The arachidonic acid pathway and the secretion of progesterone (P) and hCG by cultured human choriocarcinoma (JEG-3) cells. Proceedings of the 29th Annual Meeting of the Society for Gynecologic Investigation, Dallas, TX, March 24–27, 1982. Abstract 221, 1982.
26. Jaffe, M. L., Munson, P. J., and Rodbard, D. Four-Parameter Logistic Curve-Fitting Program for the TI-59 Calculator (Technical Report). Bethesda, MD: NIH, 1979.
27. Jaken, S., Tashjian, A. H., Jr., and Blumberg, P. M. Characterization of phorbol ester receptors and their down-modulation in GH_4C_3 rat pituitary cells. Cancer Res., *41:* 2175–2181, 1981.
28. Jones, P. B. C., and Hsueh, A. J. W. Pregnenolone biosynthesis by cultured rat granulosa cells: modulation by follicle-stimulating hormone and gonadotropin-releasing hormone. Endocrinology, *111:* 713–721, 1982.
29. Jones, P. B. C., and Hsueh, A. J. W. Direct stimulation of ovarian progesterone-metabolizing enzyme by gonadotropin-releasing hormone in cultured granulosa cells. J. Biol. Chem., *256:* 1248–1254, 1981.
30. Jones, P. B. C., and Hsueh, A. J. W. Regulation of ovarian 3β-hydroxysteroid dehydrogenase activity by gonadotropin-releasing hormone and follicle-stimulating hormone in cultured rat granulosa cells. Endocrinology, *110:* 1663–1671, 1982.
31. Jones, P. B. C., Welsh, T. H., Jr., and Hsueh, A. J. W. Regulation of ovarian progestin production by epidermal growth factor in cultured rat granulosa cells. J. Biol. Chem., *257:* 11268–11273, 1982.
32. Levine, L., and Hassid, A. Effects of phorbol-12,13-diesters on prostaglandin production and phospholipase activity in canine kidney (MDCK) cells. Biochem. Biophys. Res. Commun., *79:* 477–484, 1977.
33. Lotem, J., and Sachs, L. Regulation of normal differentiation in mouse and human myeloid leukemic cells by phorbol esters and the mechanism of tumor promotion. Proc. Natl. Acad. Sci. U. S. A., *76:* 5158–5162, 1979.
34. Lowe, M. E., Pacifici, M., and Holtzer, H. Effects of phorbol-12-myristate-13-acetate on the phenotypic program of cultured chondroblasts and fibroblasts. Cancer Res., *38:* 2350–2356, 1978.
35. Lowitt, S., Farese, R. V., Sabir, M. A., and Root, A. W. Rat Leydig cell phospholipid content is increased by luteinizing hormone and 8-bromo-cyclic AMP. Endocrinology, *111:* 1415–1417, 1982.
36. Malaisse, W. J., Sener, A., Herchuelz, A., Carpinelli, A. R., Poloczek, P., Winand, J., and Castagna, M. Insulinotropic effect of the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate in rat pancreatic islets. Cancer Res., *40:* 3827–3831, 1980.
37. Murono, E. P., and Payne, A. H. Testicular maturation in the rat. *In vivo* effect of gonadotropins on steroidogenic enzymes in the hypophysectomized im-

mature rat. Biol Reprod., *20:* 911–917, 1979.
38. Naor, Z., and Catt, K. J. Mechanism of action of gonadotropin-releasing hormone. Involvement of phospholipid turnover in luteinizing hormone release. J. Biol. Chem., *256:* 2226–2229, 1981.
39. Niedel, J. E., Kuhn, L. J., and Vandenbark, G. R. Phorbol diester receptor copurifies with protein kinase-C. Proc. Natl. Acad. Sci. U. S. A., *80:* 36–40, 1983.
40. Osborne, C. K., Hamilton, B., Hover, M., and Ziegler, J. Antagonism between epidermal growth factor and phorbol ester tumor promoters in human breast cancer cells. J. Clin. Invest., *67:* 943–951, 1981.
41. Osborne, R., and Tashjian, A. H., Jr. Tumor-promoting phorbol esters affect production of prolactin and growth hormone by rat pituitary cells. Endocrinology, *108:* 1164–1170, 1981.
42. Osborne, R., and Tashjian, A. H., Jr. Modulation of peptide binding to specific receptors on rat pituitary cells by tumor-promoting phorbol esters: decreased binding of thyrotropin-releasing hormone and somatostatin as well as epidermal growth factor. Cancer Res., *42:* 4375–4381, 1982.
43. Peterfreund, R. A., and Vale, W. W. Phorbol diesters stimulate somatostatin secretion from cultured brain cells. Endocrinology, *113:* 200–208, 1983.
44. Schomberg, D. W., Stouffer, R. L., and Tyrey, L. Modulation of progesterone secretion in ovarian cells by 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone): a direct demonstration in monolayer culture. Biochem. Biophys. Res. Commun., *68:* 77–81, 1976.
45. Shoyab, M., and Todaro, G. J. Specific high-affinity cell membrane receptors for biologically active phorbol and ingenol esters. Nature (Lond.), *288:* 451–455, 1980.
46. Shoyab, M., and Todaro, G. J. Partial purification and characterization of a binding protein for biologically active phorbol and ingenol esters from murine sera. J. Biol. Chem., *257:* 439–445, 1982.
47. Shoyab, M., Warren, T. C., and Todaro, G. J. Isolation and characterization of an ester hydrolase active on phorbol diesters from murine liver. J. Biol. Chem., *256:* 12529–12534, 1981.
48. Smith, M. A., and Vale, W. W. Superfusion of rat anterior pituitary cells attached to Cytodex beads: validation of a technique. Endocrinology, *107:* 1425–1431, 1980.
49. Snedecor, G. W., and Cochran, W. G. Statistical Methods, Ed. 6 Ames, IA: Iowa State University Press, 1967.
50. Van Duuren, B. L., and Sivak, A. Tumor-promoting agents from *Croton tiglium L.* and their mode of action. Cancer. Res., *28:* 2349–2362, 1968.
51. Virji, M. A. G., Steffes, M. W., and Estenson, R. D. Phorbol myristate acetate: effect of a tumor promoter on insulin release from isolated islets of Langerhans. Endocrinology, *102:* 706–711, 1978.
52. Wang, C., Hsueh, A. J. W., and Erickson, G. F. Induction of functional prolactin receptors by follicle-stimulating hormone in rat granulosa cells *in vivo* and *in vitro*. J. Biol. Chem., *254:* 11330–11336, 1980.
53. Welsh, T. H., Jr., and Hsueh, A. J. W. Mechanism of the inhibitory action of epidermal growth factor on testicular androgen biosynthesis *in vitro*. Endocrinology, *110:* 1498–1506, 1982.
54. Yamasaki, H., Fiback, E., Nudel, U., Weinstein, I. B., Rifkind, R. A., and Marks, P. A. Tumor promoters inhibit spontaneous and induced differentiation in murine erythroleukemia cells in culture. Proc. Natl. Acad. Sci. U. S. A., *74:* 3451–3455, 1977.