An inhibitory role for the protein kinase C pathway in ovarian steroidogenesis

Studies with cultured swine granulosa cells

Johannes D. VELDHUIS* and Lawrence M. DEMERS†

*Division of Endocrinology, Department of Internal Medicine, Box 202, University of Virginia School of Medicine, Charlottesville, VA 22908, and †Department of Pathology, Milton S. Hershey Medical Center, Pennsylvania State University School of Medicine, Hershey, PA 17033, U.S.A.

We have used primary cultures of swine granulosa cells to investigate the regulatory role of the protein kinase C pathway in the ovary. In this system, we observed the following. (1) Swine granulosa cells bound [³H]phorbol 12,13-dibutyrate ([³H]PDB) specifically with high affinity [apparent K_i for 12-*O*-tetradecanoylphorbol 13-acetate (TPA) = 3.1 (2.1–4.7) nM] and low capacity [0.68 (0.34–0.99) pmol/10⁷ cells]. (2) The cytosol of granulosa cells contained functionally active protein kinase C capable of phosphorylating distinct proteins in response to stimulation with active phorbol ester. (3) TPA and PDB induced dose-dependent inhibition (> 85%) of follicle-stimulating-hormone (FSH)-stimulated progesterone production. Half-maximally inhibitory concentrations were 0.10 and 0.75 nM for TPA and PDB respectively, whereas phorbol analogues that do not activate protein kinase C were not inhibitory. (4) TPA did not impede cyclic AMP generation in response to FSH, cholera toxin or forskolin acutely (within 48 h), but did inhibit the stimulatory effects of 8-bromo cyclic AMP, insulin and oestradiol on progesterone biosynthesis. (5) In the presence of maximally effective concentrations of 25-hydroxy-, 20 α -hydroxy- or 22*R*-hydroxy-cholesterol as exogenous sterol substrates for cholesterol side-chain cleavage, treatment with TPA suppressed pregnenolone, progesterone and 20 α -hydroxypregn-4-en-3-one biosynthesis by more than 80%. (6) The inhibitory effects of phorbol esters were not attributable to non-specific cytotoxicity, since prostaglandin F_{2 α} production increased in the same cultures and aromatization of exogenously supplied testosterone to oestradiol was not suppressed. (7) In intact granulosa cells, the effects of phorbol esters were mimicked by a synthetic non-diterpene diacylglycerol, 1-octanoyl-2-acetyl-glycerol, and the tumour promoter, mezerein, which specifically activates protein kinase C. We conclude that swine granulosa cells contain specific high-affinity receptors for phorbol esters that are functionally coupled to protein phosphorylation. Moreover, treatment with phorbol esters or non-phorbol activators of protein kinase C results in selective inhibition of cholesterol side-chain cleavage activity without impairing cyclic AMP generation or oestrogen biosynthesis.

INTRODUCTION

A Ca²⁺-sensitive, lipid-activated, protein kinase (protein kinase C) modulates the expression of differentiated function in a variety of neoplastic and non-neoplastic cells (Takai *et al.*, 1979; Kuo *et al.*, 1980). This enzyme is stimulated by endogenous diacylglycerols or exogenous diterpene compounds such as phorbol esters (Takai *et al.*, 1979; Kuo *et al.*, 1980). Recent studies suggest that protein kinase C is present in gonadal tissue and may play a role in modulating Leydig-cell or granulosa-cell function (Kimura *et al.*, 1984; Mukhopadhyay *et al.*, 1984; Welsh *et al.*, 1984). Although work to date has suggested one or more important roles for protein kinase C in modulating the steroidogenic activity of gonadal cells, available data differ regarding the exact nature of the actions of protein kinase C on the steroidogenic pathway. For example, in Leydig cells, phorbol esters inhibited gonadotropin-stimulated steroidogenesis, apparently by antagonizing cyclic AMP generation rather

than by inhibiting the actions of available cyclic AMP (Mukhopadhyay & Schumacher, 1985). In contrast, in rat granulosa cells, the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) stimulated basal progesterone accumulation but inhibited FSH- and cyclic AMP-stimulated steroidogenesis (Welsh *et al.*, 1984; Kawai & Clark, 1985).

In the present studies we have examined the mechanism(s) of phorbol action *in vitro* by using swine granulosa cells, whose steroidogenic pathway is highly responsive to perturbations in calcium concentrations (Veldhuis & Hammond, 1981; Veldhuis & Klase, 1982). We have employed this system to: (1) evaluate the properties of high-affinity specific phorbol-ester binding to granulosa cells; (2) examine the ability of phorbol esters to stimulate the phosphorylation of distinct proteins in these cells; (3) test the site(s) of action of phorbol esters on the steroidogenic pathway; and (4) assess the specificity of these effects in relation to progesterone biosynthesis.

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDB, phorbol 12,13-dibutyrate; PDA, phorbol 12,13-diacetate; PM, phorbol 13-monoacetate; 4 α -PDD, 4 α -phorbol 12,13-didecanoate; FSH, follicle-stimulating hormone (follicotropin).

† To whom correspondence and reprint requests should be sent.

MATERIALS AND METHODS

Methods

Swine granulosa cells were harvested from 1–5 mm follicles by fine-needle aspiration as described previously (Veldhuis & Hammond, 1981). Cells were cultured in 1% (v/v) fetal-bovine serum (Gibco) with indicated concentrations of effector substances. Unless designated otherwise, cultures were harvested at 48 h for the measurement of total progesterone content in cells combined with medium. FSH was dissolved in saline, and phorbol compounds were dissolved in ethanol, before addition to culture medium (final ethanol concentration < 0.1%). Control cultures received an equivalent volume of diluent.

Cellular binding of [³H]phorbol 12,13-dibutyrate (PDB) was examined in granulosa-cell cultures that had been established in 1% bovine-fetal serum for 3 days and then washed three times in serum-free medium. After preliminary time-course experiments which demonstrated that binding reached equilibrium after 3–4 h at 4 °C, further experiments were conducted at 4 °C for 4 h with indicated concentrations of [³H]PDB and competitors. The subsequent equilibrium competition curves were analysed by non-linear least-squares curve-fitting. The precision of each fitted parameter was determined from observed experimental variance, so that results were expressed as mean estimates with corresponding 95% confidence limits (Johnson, 1983). The K_i was calculated from the half-maximally effective inhibitory concentration, ID_{50} , and the K_d , using the relationship:

$$K_i = ID_{50} / (1 + L/K_d)$$

where L is the concentration of radioligand.

The ability of phorbol esters to activate protein kinase C in intact granulosa cells was assessed as described for EL4 mouse thymoma cells (Sando & Young, 1983). Before addition of phorbol ester, granulosa cells were exposed to 100 μ Ci of [³²P]orthophosphate/ml for 45 min to label endogenous ATP stores. Cultures of granulosa cells (1.8×10^7 /10 cm dish) were then treated with 0.1 or 1.0 μ M-TPA for 30 min, harvested mechanically by scraping into phosphate-buffered saline (0.9%, pH 7.4), centrifuged rapidly, and resuspended in water and NaF (50 mM) to promote rapid cell lysis. A 50 μ g portion of cytosol protein was then subjected to SDS/10%-(w/v)-polyacrylamide-gel electrophoresis followed by autoradiography (Veldhuis & Hewlett, 1985).

Cyclic AMP production was measured by specific radioimmunoassay as described (Veldhuis & Klase, 1982). Oestradiol was assayed by radioimmunoassay after Celite-column chromatography, and 20 α -hydroxypregn-4-en-3-one, progesterone and pregnenolone by radioimmunoassay after extraction with organic solvents, as reported previously (Veldhuis & Klase, 1982).

Materials

Culture media were from Gibco (Grand Island, NY, U.S.A.), and oestradiol, 25-hydroxycholesterol and other chemicals from Sigma Chemical Corp. (St. Louis, MO, U.S.A.). [³H]PDB [20-³H(n)]phorbol 12,13-dibutyrate; specific radioactivity 30.8 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.) Ovine FSH (NIH-oFSH-S16) was provided by the National Hormone Distribution Office (Bethesda, MD, U.S.A.).

Fig. 1. Equilibrium competition curves for the binding of ([³H]PDB) to swine granulosa cells (1×10^7) in monolayer culture

Granulosa cells were incubated with [³H]PDB (5 nM) and with the indicated concentrations of competitors for 4 h at 4 °C. The subsequent competition curves were analysed by non-linear least-squares curve-fitting (see under 'Methods'). Results are means $\pm$ S.E.M. for three independent determinations representing three experiments; 100% [³H]PDB binding represented 2419 ± 25 c.p.m./ 10^7 granulosa cells in these experiments.

RESULTS

Swine granulosa cells contained specific, low-capacity, high-affinity and saturable binding sites for phorbol esters as revealed by equilibrium-competition-curve experiments performed in cultures incubated with [³H]PDB (5 nM) at 4 °C for 4 h in the presence or absence of increasing concentrations of unlabelled TPA (1–1000 nM) (Fig. 1). Unlabelled TPA effectively inhibited of [³H]PDB binding to intact granulosa cells with an apparent ID_{50} of 3.74 nM (2.5–5.6 nM) (95% confidence limits) as estimated from three separate determinations. The corresponding K_i for TPA calculated from this ID_{50} and from the K_d for [³H]PDB (see below) was 3.12 (2.09–4.68) nM. When PDB was utilized both as ligand ([³H]PDB) and competitor, the resulting equilibrium competition curves yielded an estimated K_d of 25.4 nM (17.8–45.5 nM), and an estimated maximal specific binding capacity of 0.68 (0.34–0.99) pmol/ 10^7 granulosa cells. This binding was specific in that it was not inhibited by less active compounds, namely 4 α -phorbol 12,13-didecanoate (4 α -PDD) or pure phorbol (unesterified base) (Fig. 1).

To assess the ability of phorbol esters to promote the phosphorylation of specific ovarian proteins, cell-free cytosol was incubated in the presence of excess EGTA (Ca^{2+} -depleted), the classical ovarian effector agent, cyclic AMP (100 μ M), Ca^{2+} (free Ca^{2+} concentration $\approx$ 400 μ M), and the combination of Ca^{2+} , phosphatidylserine (96 μ g/ml), and 1,2-dioleoylglycerol (3.2 μ g/ml) to activate protein kinase C or TPA (10 or 100 nM). The cytosolic proteins were then subjected to SDS/polyacrylamide-gel electrophoresis and autoradiography (Fig. 2). Under conditions presumed to activate protein kinase C, incorporation of ³²P was observed in multiple bands of estimated molecular mass 18, 36, 47,

Fig. 2 Autoradiogram of SDS/polyacrylamide-gel electrophoretogram of luteal cytosol incubated with [³²P]ATP and stimulated *in vitro*

Lane A, control (1.0 mM excess EGTA); lane B, cyclic AMP (100 μM); Lane C, Ca²⁺ stimulation (free [Ca²⁺] 400 μM); lane D, stimulation with 400 μM free Ca²⁺ + phosphatidylserine (96 μg/ml) and 1,2-dioleoylglycerol (3.2 μg/ml); and lanes E and F, stimulation with TPA (100 nM) and TPA (10 nM) in the presence of 400 μM free Ca²⁺ and phosphatidylserine (96 μg/ml). All reaction mixtures contained 0.1 mM-ATP and 50 μg of cytosol protein. In the far-left lane(s), marks designate *M_r* standards.

55, 66, 110 and 120 kDa. Similarly migrating bands of phosphorylated proteins were also observed when luteal cytosol was stimulated with either the combination of Ca²⁺, phosphatidylserine and dioleoylglycerol, or TPA alone.

To test the effects of phorbol compounds on

Fig. 3. Dose-dependent inhibition by TPA of basal (●) and FSH-stimulated (*) progesterone production by cultured swine granulosa cells

Granulosa cells (2×10^6) were cultured for 48 h in the presence or absence of FSH (200 ng/ml) with or without increasing concentrations of the phorbol ester TPA. The total content of progesterone was measured in cells combined with medium. Results are means $\pm$ S.E.M. (four cultures) from two independent experiments.

granulosa-cell function, cultures were treated with TPA or control solvent in the presence or absence of a maximally effective dose of FSH (200 ng/ml). The time course of the inhibitory effect of TPA (3 ng/ml) is given in Table 1. Thus, in the remaining experiments, a 48 h incubation was used. As shown in Fig. 3, in the absence of FSH, TPA effectively inhibited basal progesterone production in a dose-dependent fashion with an ID₅₀ of 0.048 ng/ml (95% confidence limits 0.025–0.11 ng/ml). TPA similarly suppressed FSH-stimulated progesterone production with an ID₅₀ of 0.064 ng/ml (0.042–0.13 ng/ml). Under these conditions, the less active

Table 1. Time-dependent influences of the phorbol ester TPA on FSH-stimulated progesterone production by swine granulosa cells

Granulosa cells (2×10^6) were incubated (see the Materials and methods section) for the indicated times with or without FSH (200 ng/ml) or TPA (3 ng/ml), and the subsequent mass of progesterone in medium determined by radioimmunoassay. Data are means $\pm$ S.D., *n* = 4 determinations. Differing superscripts (a–d) denote significantly different means by analysis of variance.

Time (h)	Progesterone production (ng/10 ⁶ cells)			
	Control	FSH	TPA alone	FSH + TPA
3	2.8 $\pm$ 0.5 ^a	8.3 $\pm$ 1.2 ^b	2.1 $\pm$ 0.3 ^a	6.6 $\pm$ 0.4 ^b
7	3.6 $\pm$ 0.3 ^a	10.8 $\pm$ 0.5 ^b	3.3 $\pm$ 0.3 ^a	7.5 $\pm$ 0.6 ^c
24	7.1 $\pm$ 0.4 ^a	15.5 $\pm$ 0.8 ^b	5.2 $\pm$ 0.5 ^a	8.4 $\pm$ 0.4 ^{a,c}
48	7.5 $\pm$ 0.7 ^a	18.0 $\pm$ 1.6 ^b	5.6 $\pm$ 0.3 ^c	8.8 $\pm$ 0.6 ^{a,d}

Table 2. Influence of the phorbol ester TPA (10 ng/ml) on effector- and hormonally-stimulated progesterone production by cultured swine granulosa cells

Results are means $\pm$ S.E.M. for four independent determinations.

Conditions	Progesterone production (ng/48 h per 2×10^6 cells)	
	Control	+ TPA
Basal	8.9 ± 0.8	$6.5 \pm 0.3^\dagger$
Forskolin (30 μ M)	1650 ± 21	$23 \pm 1.0^\dagger$
Cholera toxin (1 μ g/ml)	440 ± 26	$24 \pm 0.9^\dagger$
Oestradiol (1 μ g/ml)	21 ± 1.1	$6.7 \pm 0.2^\dagger$
Insulin (1 μ g/ml)	328 ± 5	$10 \pm 0.5^\dagger$

$^\dagger P < 0.05$ versus control.

phorbol compound (PDB) exhibited an ID_{50} of 0.49 ng/ml (0.29–1.0 ng/ml) in inhibiting FSH-stimulated progesterone production, and an ID_{50} of 0.38 ng/ml (0.21–0.75 ng/ml) for basal progesterone production; for the plant-derived tumour promoter, mezerein, the ID_{50} was 0.23 (0.13–0.49) ng/ml basally and 0.25 (0.08–0.96) ng/ml in inhibiting FSH-stimulated progesterone production. The corresponding ID_{50} values for phorbol 12,13-diacetate (PDA) were 16.7 ng/ml (basal) and 22.1 ng/ml (FSH-treated). For comparison, the respective K_i and K_d estimates for TPA and PDB binding as assessed with [3 H]PDB (above) were 1.9 and 12.8 ng/ml. Notably, phorbol esters that do not activate protein kinase C were uniformly ineffective ($ID_{50} > 30$ ng/ml) in inhibiting progesterone biosynthesis by swine granulosa cells [n = eight experiments using PM (phorbol 13-monoacetate), pure phorbol base and 4 α -phorbol 12,13-didecanoate]. Thus the rank order of efficacy was TPA > PDG $\gg$ PDA $\gg$ PM, phorbol base or 4 α -PDD.

The mechanisms subserving the inhibitory effect of phorbol esters on basal and FSH-stimulated progesterone production were evaluated further by testing the ability of TPA to inhibit the stimulatory effects of cholera toxin or forskolin (Table 2). TPA (10 ng/ml) markedly inhibited the stimulatory effects of forskolin and cholera toxin on total progesterone production ($P < 0.001$). However, TPA did not suppress the ability of FSH, forskolin or cholera toxin to stimulate short-term (48 h) total cyclic AMP accumulation (Table 3). In other experiments, TPA did not inhibit time-dependent (3–48 h) cellular or medium accumulation of cyclic AMP basally or in response to FSH (results not shown). The latter observations suggest that short-term inhibitory effects of TPA are mediated by mechanisms independently of effector-stimulated cyclic AMP generation *per se*. When incubations were extended to 96 h, TPA inhibited FSH-stimulated cyclic AMP production by granulosa cells in a dose-related (1, 3, 10 ng/ml) fashion. However, at various earlier times (3, 7, 24 and 48 h), no inhibitory effects of TPA are observable on basal or FSH-stimulated cyclic AMP production. This hypothesis was tested by assessing the ability of TPA to inhibit the stimulatory effects of insulin and oestradiol, since these hormones are not known to operate via the cyclic AMP pathway. Our results in four experiments indicated that TPA also significantly suppressed the stimulatory actions of

Table 3. Lack of influence of the phorbol ester TPA on short-term (48 h) FSH-stimulated cyclic AMP generation by swine granulosa cells

Results are means $\pm$ S.E.M. for four independent determinations confirmed in these experiments. Total cyclic AMP accumulation was measured in cells combined with medium after incubation of monolayer cultures (2×10^6 granulosa cells) for 48 h in the presence of the indicated effectors and 1-isobutyl-3-methylxanthine (0.25 mM) to inhibit phosphodiesterase activity.

Treatment conditions	Cyclic AMP generation (pmol/48 h per 10^6 cells)
Basal	1.31 ± 0.08
Basal + TPA (1 ng/ml)	1.34 ± 0.07
Basal + TPA (10 ng/ml)	1.16 ± 0.07
FSH	$8.15 \pm 0.70^*$
FSH + TPA (1 ng/ml)	$8.75 \pm 0.46^*$
FSH + TPA (10 ng/ml)	$9.98 \pm 1.3^*$
Forskolin (30 μ M)	$10.0 \pm 0.52^*$
Forskolin + TPA (10 ng/ml)	$10.4 \pm 0.27^*$
Cholera toxin (1 μ g/ml)	$8.42 \pm 0.24^*$
Cholera toxin + TPA (10 ng/ml)	$8.97 \pm 0.20^*$

* $P < 0.01$ versus basal.

insulin and oestradiol on progesterone biosynthesis (Table 2).

On the basis of the preceding results, we tested the ability of TPA to inhibit progesterone production stimulated by exogenously supplied cyclic AMP or sterol substrate. As shown in Table 4, TPA (10 ng/ml) significantly inhibited progesterone production in response to stimulation with 8-bromocyclic AMP or exogenous sterol substrate (25-hydroxycholesterol). These inhibitory effects on progesterone accumulation were associated with diminished production of progesterone's reduced metabolite, 20 α -hydroxypregn-4-en-3-one (20 α -dihydroprogesterone) as well. The latter observation indicates that TPA does not selectively increase progesterone's catabolism to its 20 α -reduced metabolite and thereby indirectly decrease total progesterone accumulation.

When granulosa cells were exposed to maximally effective concentrations of 25-hydroxycholesterol for 4 h in the presence of trilostane (150 μ M) to test functional cholesterol side-chain-cleavage activity, prior TPA (10 ng/ml) treatment resulted in significant inhibition of pregnenolone production (Table 4, $P < 0.01$ treatment effect). This action was not shared by the less active phorbol derivative, PM (4 β -phorbol 13-monoacetate). In addition, treatment with 22 R - and 20 α -hydroxycholesterol also failed to overcome the inhibitory effect of TPA on progesterone biosynthesis (results not shown; three experiments). In contrast, when granulosa cells were treated with a maximally effective concentration of pregnenolone (25 μ g/ml), the production of progesterone was unimpaired in the presence of phorbol esters: control, 97.5 ± 5.3 ng of progesterone/48 h, versus TPA (10 ng/ml), 99.0 ± 5.7 ng of progesterone/48 h (not significant). These observations suggest that inhibition of progesterone biosynthesis occurs in large part at the level

Table 4. Inhibitory effects of the phorbol ester TPA (10 ng/ml) on effector-stimulated synthesis of progesterone, 20 α -hydroxypregn-4-en-3-one and pregnenolone

Production of:	Conditions	Control	+ TPA
(a) Progesterone (ng/48 h per 2×10^6 cells)	Basal	51 $\pm$ 0.9	37 $\pm$ 3.6*
	8-Bromo cyclic AMP		
	0.3 mM	197 $\pm$ 4.4	59 $\pm$ 3.9*
	3.0 mM	265 $\pm$ 5.7	63 $\pm$ 28 *
	25-Hydroxycholesterol		
	1 μ g/ml	253 $\pm$ 5.2	84 $\pm$ 6.7*
	25 μ g/ml	288 $\pm$ 13	45 $\pm$ 2.5*
(b) 20 α -Hydroxypregn-4-en-3-one (ng/48 h per 2×10^6 cells)	Basal	0.72 $\pm$ 0.15	0.31 $\pm$ 0.07*
	8-Bromo cyclic AMP (3.0 mM)	2.3 $\pm$ 0.38	0.45 $\pm$ 0.05*
	25-Hydroxycholesterol (25 μ g/ml)	1.6 $\pm$ 0.16	0.41 $\pm$ 0.07*
(c) Pregnenolone (ng/4 h per 2×10^6 cells)†	Basal	41 $\pm$ 60	12 $\pm$ 4.5*

* $P < 0.05$ versus corresponding control.

† Cells were cultured for 4 h in the presence of 25-hydroxycholesterol (25 μ g/ml) and trilostane (150 μ M) to test functional cholesterol side-chain-cleavage activity.

Fig. 4. The synthetic diacylglycerol 1-octanoyl-2-acetylgllycerol (OAG) acutely inhibits FSH-stimulated progesterone production

Swine granulosa cells (2×10^6) were cultured for 2 h in the presence of the indicated concentrations of 1-octanoyl-2-acetylgllycerol. The total content of progesterone was determined in cells combined with medium. FSH was added at a concentration of 200 ng/ml where indicated. Results are otherwise as presented in the legend of Fig. 3. ■, Control; ▨, + FSH.

of cholesterol side-chain cleavage *per se* (Falke *et al.*, 1975), with consequent significant suppression of pregnenolone, progesterone and 20 α -hydroxypregn-4-en-3-one production.

The ability of phorbol esters to inhibit progestin biosynthesis was not related exclusively to the diterpine structure of the compounds, since the synthetic diacylglycerol 1-oleoyl-2-acetylgllycerol also significantly suppressed FSH-stimulated progesterone production within 2 h (Fig. 4).

The effects of phorbol esters were not attributable to non-specific cytotoxicity for several reasons. First, TPA (30 ng/ml) had no inhibitory effect on the cellular conversion of exogenously supplied testosterone into oestradiol (Fig. 5). Secondly, TPA (alone and in combination with FSH) significantly stimulated the production of prostaglandin $F_{2\alpha}$ in the same cultures in which progestin synthesis was impeded: namely from a basal rate (pg/48 h per 2×10^6 cells) of 1030 ± 33 (control) to 1750 ± 68 (FSH), 3780 ± 190 (TPA, and 5100 ± 230 (TPA + FSH). ($P < 0.01$ treatment effects.) Moreover, treatment with phorbol compounds resulted in no change in the cellular content of protein or DNA, in cell number or in cell viability (assessed by the exclusion of Trypan Blue).

DISCUSSION

The present studies using [3 H]PDB document high-affinity, specific and saturable binding of this radiolabelled phorbol ester to intact granulosa cells. Our results are in accord with observations in non-steroidogenic tissues, where similar K_d estimates for PDB binding have been reported (Horowitz *et al.*, 1981; Ileki & Beneviste, 1985; Solanki & Slaga, 1981). Moreover, the rank order of potency of binding for TPA, PDB and the inactive compounds 4 α -phorbol-12,13-didecanoate and pure

Fig. 5. Lack of influence of TPA on testosterone's aromatization to oestradiol by cultured swine granulosa cells

Monolayer cultures of pig granulosa cells (2×10^6) were exposed to increasing concentrations of testosterone (as indicated) in the presence of either control solvent (0.1% ethanol) or TPA (30 ng/ml). After 48 h, the total cellular and medium content of oestradiol was determined by radioimmunoassay. Results are means $\pm$ S.E.M. for four separate determinations. $\circ$ · · · $\circ$, Control; *—*, TPA (30 ng/ml).

phorbol, conforms to the rank order of potency of these substances in activating protein kinase C in classical phorbol target tissues (Horowitz *et al.*, 1981; Ileki & Benevise, 1985; Solanki & Slaga, 1981). Thus the present data indicate that ovarian cells contain a distinct phorbol-ester receptor that presumably represents protein kinase C (Horowitz *et al.*, 1981; Ileki & Benevise, 1985; Solanki & Slaga, 1981).

In cell-free incubations, phorbol-ester treatment of ovarian cytosol resulted in increased phosphorylation of distinct proteins assessed by SDS/polyacrylamide-gel electrophoresis. Cytosolic proteins were also phosphorylated when Ca^{2+} , phospholipid and dioleoylglycerol were added directly to cytosol. Although the exact identity of these cytosolic proteins that are phosphorylated in response to TPA or the combination of Ca^{2+} , dioleoylglycerol and phospholipid are not yet known, the present observations indicate that swine ovarian cytosol contains endogenous substrates for protein kinase C.

The functional responses of granulosa cells to treatment with phorbol esters included consistent suppression of basal, FSH-, forskolin- and cholera-toxin-stimulated progesterone biosynthesis. These inhibitory effects were not a consequence of suppression of basal or effector-stimulated cyclic AMP production, at least over 3–48 h of observation. Rather, phorbol esters inhibited the steroidogenic action of exogenously supplied 8-bromocyclic AMP and the stimulatory effects of insulin

and oestradiol, which are believed to activate steroidogenesis without directly altering cyclic AMP production. Such observations suggest that the acute inhibitory action of phorbol esters is expressed distal to, or independently of, cyclic AMP biosynthesis. This inference is in accord with that recently suggested in rat granulosa cells (Shinohara *et al.*, 1985).

The locus of inhibition of steroidogenesis by phorbol esters appeared to involve predominantly the cholesterol side-chain-cleavage step, since: (1) phorbol compounds inhibited the biosynthesis of all measured progestins; (2) TPA did not impair progesterone production when pregnenolone was supplied exogenously; and, (3) in the presence of exogenous soluble sterol substrates for cholesterol side-chain cleavage, phorbol esters still suppressed pregnenolone biosynthesis. Further studies will be required to ultimately determine the exact constituent(s) of the cholesterol side-chain-cleavage reaction (e.g., cytochrome $P-450_{\text{sc}}$, adrenodoxin etc.) affected by phorbol esters.

The actions of phorbol esters were not dependent upon the diterpene structure of these compounds, but rather presumably reflected their mimicry of diacylglycerols. Thus, the synthetic diacylglycerol 1-oleoyl-2-acetyl-glycerol also significantly suppressed FSH-stimulated progesterone production. This compound is a potent activator of protein kinase C (Kupchan & Baxter, 1974; de Courcelles *et al.*, 1984). Moreover, inhibition of progestin biosynthesis by phorbol esters was not associated with evidence of cellular toxicity, in that cellular aromatase activity was not decreased, whereas synthesis of prostaglandin $F_{2\alpha}$ was increased.

In summary, we have observed that swine granulosa cells contain specific, high-affinity and saturable phorbol-ester receptors that are functionally coupled to the phosphorylation of distinct cytosolic proteins. Phorbol esters inhibit progesterone biosynthesis specifically in a dose- and time-dependent fashion, with a rank order of potency that parallels activation of protein C. Phorbol effects are mimicked by synthetic diacylglycerol and the tumour promoter mezerein, and are expressed to a significant degree at the level of cholesterol side-chain cleavage. These results suggest that protein kinase C modulates steroid-hormone biosynthesis in an inhibitory manner in swine granulosa cells. Such an inhibitory pathway contrasts with the stimulatory responses that seem to be subserved by cyclic AMP and Ca^{2+} /calmodulin effectors in the ovary (Veldhuis & Klase, 1982).

We thank Ms. Chris McNett for the skilful preparation of the manuscript, the Gwaltney-Smithfield Packing Corporation for providing swine ovaries, Ms. Paula P. Azimi for the artwork, and Dr. Joseph Larner and Dr. Jerome F. Strauss III for helpful discussions, Ms. Paula Azimi, Ms. Diana Juchter, Mr. James Garmey and Mr. Walter May for excellent technical assistance, and Dr. Julianne J. Sando for her expert advice and assistance in the radioligand and protein-electrophoresis studies. This work was supported in part by a National Institutes of Health Grant no. R 01 HD16806 and RCDA No. 1 K04 HD 00634 (to J.D.V.).

REFERENCES

- de Courcelles, D. D. C., Roevens, P. & Van Belle, H. (1984) *Biochem. Biophys. Res. Commun.* **123**, 589–595
- Falke, H. E., Degenhart, H. J., Abeln, G. J. A. & Visser, H. K. A. (1975) *Mol. Cell. Endocrinol.* **3**, 375–383

- Horowitz, A. D., Greenebaum, E. & Weinstein, I. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2315–2319
- Ilekis, J. & Benveniste, R. (1985) *Endocrinology* (Baltimore) **116**, 2400–2409
- Johnson, M. L. (1983) *Biophys. J.* **44**, 101–106
- Kawai, Y. & Clark, M. R. (1985) *Endocrinology* (Baltimore) **116**, 2320–2326
- Kimura, K., Katoh, N., Sakurada, K. & Kubo, S. (1984) *Endocrinology* (Baltimore) **115**, 2391–2399
- Kuo, J. F., Anderson, R. G., Wise, B. C., Mackerlova, L., Solomonsson, I., Brackett, N. L., Katoh, N., Shoji, M. & Wrenn, R. W. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7039–7043
- Kupchan, S. M. & Baxter, R. L. (1974) *Science* **187**, 652–653
- Mukhopadhyay, A. K. & Schumacher, M. (1985) *FEBS Lett.* **187**, 56–60
- Mukhopadhyay, A. K., Bohnet, H. G. & Leidenberger, F. A. (1984) *Biochem. Biophys. Res. Commun.* **119**, 1062–1067
- Sando, J. J. & Young, M. C. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2642–2646
- Shinohara, O., Knecht, M. & Catt, K. J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8518–8522
- Solanki, V. & Slaga, T. J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2549–2553
- Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979) *J. Biol. Chem.* **254**, 3692–3695
- Veldhuis, J. D. & Hammond, J. M. (1981) *Biochem. J.* **196**, 795–801
- Veldhuis, J. D. & Klase, P. A. (1982) *Biochem. J.* **202**, 381–386
- Veldhuis, J. D. & Hewlett, E. L. (1985) *Biochem. Biophys. Res. Commun.* **131**, 1168–1174
- Welsh, T. H., Jones, P. B. C. & Hsueh, A. J. W. (1984) *Cancer Res.* **44**, 885–892

Received 23 January 1986/19 May 1986; accepted 24 June 1986