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# Regulation of the Corpus Luteum by Protein Kinase C I. Phosphorylation Activity and Steroidogenic Action in Large and Small Ovine Luteal Cells[1]

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## ABSTRACT

*The activity and steroidogenic action of protein kinase C were evaluated in small and large steroidogenic ovine luteal cells. Protein kinase C activity (per mg protein) was threefold greater in large than in small luteal cells, whereas protein kinase A activity was similar in the two cell types. Phorbol 12-myristate 13-acetate (PMA) activated protein kinase C in luteal cells as demonstrated by membrane association of 91% of available protein kinase C within 15 min of PMA treatment. Longer treatments with PMA produced cells with low protein kinase C activity (protein kinase C-deficient cells) but did not affect cellular viability or protein kinase A activity. Activation of protein kinase C caused an acute, dose-dependent inhibition of progesterone production in unstimulated large and luteinizing hormone (LH)-stimulated small luteal cells. This inhibition by PMA appeared to be specific for protein kinase C since it was greatly attenuated in protein kinase C-deficient cells and since an inactive phorbol ester, 4α-phorbol, had no effect on luteal progesterone production. The inhibitory locus of protein kinase C action in small luteal cells appeared to be distal to the adenylate cyclase enzyme because progesterone production was inhibited similarly in cells stimulated with LH, forskolin, or dibutyryl cyclic adenosine 3′,5′-monophosphate. Cholesterol side-chain cleavage activity, as measured by metabolism of 25-hydroxycholesterol, was inhibited by PMA in large, but not in small, luteal cells. These data indicate that activation of protein kinase C specifically inhibits progesterone production in both large and small ovine luteal cells, although the intracellular mechanisms invoked appear to differ in the two cell types.*

## INTRODUCTION

Phosphorylation of proteins is an important mechanism for regulating cellular function in all cell types including luteal cells. Activation of cyclic adenosine 3′,5′-monophosphate dependent protein kinase (protein kinase A) is required for stimulation of progesterone production by small luteal cells (Hoyer and Niswender, 1986). A more recently identified protein kinase that is phospholipid-stimulated and calcium-dependent (protein kinase C) has also been detected in luteal cells (Davis and Clark, 1983; Budnik and Mukhopadhyay, 1985; Noland and Dimino, 1986; Wheeler and Veldhuis, 1987). Although the hormonal regulation of protein kinase C in luteal cells has not been fully elucidated, phosphatidylinositol metabolism, which could ultimately lead to activation of protein kinase C, appears to be stimulated by both luteinizing hormone (LH) (Davis et al., 1987a; Allen et al., 1988) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Leung et al., 1986; Davis et al., 1987b; Jacobs et al., 1987). Pharmacologically, protein kinase C can be activated by tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA). Treatment with PMA has been reported to either stimulate or inhibit production of progesterone by luteal cells from different species (Brunswig et al., 1986; Baum and Rosberg, 1987; Benhaim et al., 1987; Hansel et al., 1987; Jalkanen et al., 1987; Alila et al., 1988). The objective of the present study was to quantitate the activity of protein kinase C and protein kinase A activity in large and small ovine luteal cells and to

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evaluate the effect of treatment with PMA on steroidogenesis in these cell types.

## MATERIALS AND METHODS

### *Preparation of Cells*

Corpora lutea were collected by mid-ventral laparotomy from Western range ewes on Day 10 postestrus. Corpora lutea were decapsulated, sliced, and dissociated into single cell suspensions with 0.25% (w/v) collagenase (141 units/mg; Worthington Biochemical, Freehold, NJ) and 0.05% (w/v) deoxyribonuclease (Sigma Chemical Co., St. Louis, MO). The cells were separated by centrifugal elutriation into small (10–20 μm) and large (20–35 μm) steroidogenic luteal cell fractions (Fitz et al., 1982).

For experiments with plated cells, small ($5 \times 10^4$ cells/well) or large ($15 \times 10^3$ cells/well) cells were allowed to attach overnight onto 24-well plates (Corning, Corning, NY; #25820) in Medium 199 (GIBCO Labs, Grand Island, NY; #400-1200) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4.2 mM $NaHCO_3$, 100 IU/ml of penicillin G, 10 μg/ml streptomycin sulfate (M199), and 5% (v/v) ram serum. The following day, the cells were washed 3 times with M199 and treatments with LH (100 ng/ml; NIADDK-oLH-25), forskolin (50 μM; Calbiochem-Behring Diagnostics, La Jolla, CA; #344270), dibutyryl cAMP (dBcAMP; 10 μM; Sigma Chemical Co., #D0627), PMA (0–1000 nM; Sigma Chemical Co., #P8139), 4α-phorbol (0–1000 nM; Sigma Chemical Co., #P4888), and/or 25-hydroxycholesterol (50 μM; Sigma Chemical Co., #H1015) were initiated. Maximal stimulatory doses of LH, forskolin, dBcAMP, and 25-hydroxycholesterol were determined in preliminary experiments. Cell viability was evaluated at the end of treatments with ethidium bromide/acridine orange viability stain (Parks et al., 1979).

### *Measurement of Protein Kinase Activity*

Small ($6 \times 10^6$) or large ($2 \times 10^6$) luteal cells were incubated at 37°C in 10 ml M199 containing PMA or ethanol (vehicle for PMA). After incubation, the cells were centrifuged and washed twice with fresh Medium 199. Cells were then placed in 500 μl ice-cold distilled water containing 50 μg/ml leupeptin, lysed by repeated passage through a 25-gauge needle, and ultrasonicated for 15 s. The lysate was resuspended in 5 ml ice-cold, 20 mM tris (hydroxymethyl)aminomethane (Tris) buffer (Sigma Chemical Co., #T1503; pH 7.5) containing 5 mM ethylenediaminetetraacetate (EDTA, Sigma Chemical Co., #ED2SS), 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., #P2626), and 0.25 M sucrose (Buffer A). The cell lysate was incubated for 1 h at 4°C in Buffer A containing 0.1% Triton X-100 (Sigma Chemical Co., #T6878) and centrifuged at $28,000 \times g$ for 20 min; the supernatant was designated as containing the total protein kinase C activity since Triton X-100 solubilizes the membrane-bound protein kinase C. To collect cytosolic vs. membrane-bound protein kinase C, the original cell lysate without Triton X-100 was centrifuged at $28,000 \times g$ for 20 min. This supernatant was designated the cytosolic fraction. The pellet was resuspended in Buffer A containing 0.1% Triton X-100, incubated at 4°C for 1 h, and centrifuged at $28,000 \times g$ for 20 min; this supernatant was designated the membrane-bound fraction. All fractions remained at 4°C until measurement of kinase activity.

Protein kinase activity was quantitated by transfer of $^{32}P$ from [gamma-$^{32}P$]ATP (Amersham Corp, Arlington Heights, IL) to histone IIIS (Sigma Chemical Co., #H5505) by modification of published procedures (Noland and Dimino, 1986; Thomas et al., 1987; Wheeler and Veldhuis, 1987). The reaction mixture (total volume = 160 μl) contained 20 mM Tris (pH 7.5), 10 mM magnesium acetate (Sigma Chemical Co., #M0631), 100 μM ATP (240 cpm [$^{32}P$]ATP/pmole), 25 μg histone IIIS, and 50 μg/ml leupeptin (Boehringer-Mannheim Biochem., Indianapolis, IN). The mixture also contained different combinations of 0.75 mM $CaCl_2$, 100 μg/ml phosphatidylserine (Sigma Chemical Co., P8518), 5 μg/ml 1,2-diolein (Sigma Chemical Co., #D0138), 1.5 μM PMA, and 5 mM dBcAMP. The reaction was carried out for 3 min at 30°C and terminated by addition of 500 μl of ice-cold 25% trichloroacetic acid. Bovine serum albumin (BSA, 0.1 ml of a 0.1 M solution) and 3 ml of 5% (v/v) trichloroacetic acid were then added. The suspensions were centrifuged at $2000 \times g$ for 10 min, supernatants were discarded, and the pellets were resuspended in 0.1 ml of 0.5 M NaOH. The precipitation and resuspension procedure was repeated twice. The final resuspended pellet was placed in 4 ml PBS, and the radioactivity was determined by Cherenkov counting in a Beckman liquid scintillation counter. Protein kinase C activity was determined by subtracting the activity in the presence of calcium, phosphatidylserine, and PMA from the activity

in the presence of calcium without phosphatidylserine or PMA (Thomas et al., 1987). Protein kinase A activity was determined by measuring the difference in activity in the presence or absence of dBcAMP.

### *Measurement of Progesterone*

The concentration of progesterone in medium was quantified by radioimmunoassay (Niswender, 1973). The intra- and interassay coefficients of variation for sixteen assays were 6 and 16.5% at 0.75 ng/ml and 6 and 8% at 4 ng/ml. The limit of detection was 0.4 pg/tube.

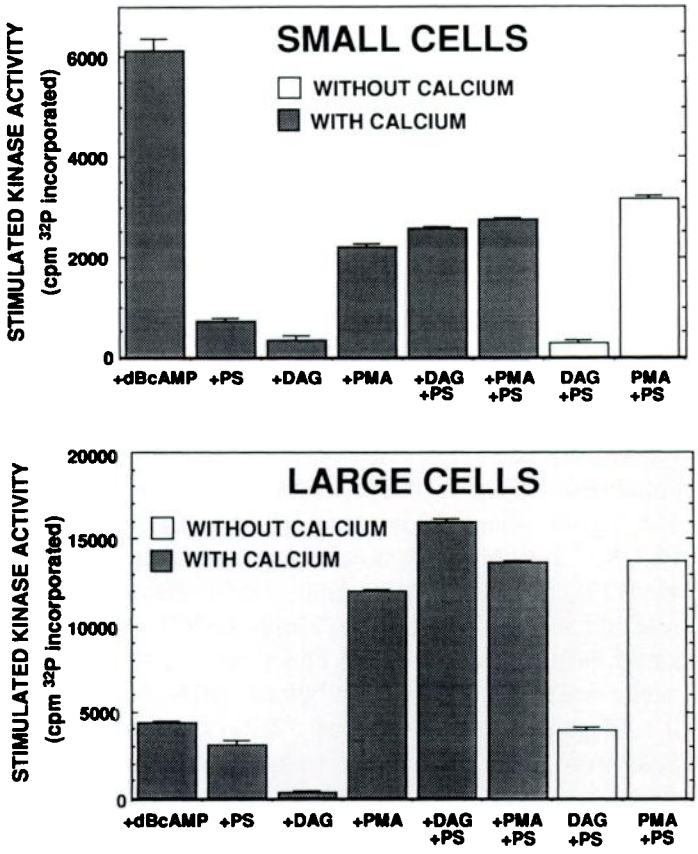


FIG. 1. The total kinase activity (mean ± SD for triplicates) in homogenates from large or small ovine luteal cells stimulated by dibutryl cyclic adenosine 3′,5′-monophosphate (*dBcAMP*; 5 mM), phosphatidylserine (*PS*; 1 μM), 1,2-diolein (*DAG*; 8 μM), phorbol 12-myristate 13-acetate (*PMA* 1 μM), and/or calcium (*with calcium* = 10 μM calcium chloride; *without calcium* = no added calcium + 0.5 μM ethylenediaminetetraacetate). The nonspecific kinase activity (background), which was subtracted from appropriate treatments, was small cells without calcium: 1900 cpm; small cells with calcium: 1106 cpm; large cells without calcium: 2300 cpm; large cells with calcium: 1476 cpm.

### *Statistics*

Overall effect of treatments was evaluated by analysis of variance for repeated measures using the Statview 512+ program (Brain Power, Inc., Calabases, CA) for the MacIntosh computer. Significant differences between treatments were evaluated with the Dunnett *t*-test (Dunnett, 1964).

## RESULTS

The effects of calcium, phospholipids, and PMA on kinase activity in extracts of small or large luteal cells are summarized in Figure 1. Maximal activation of protein kinase C by PMA occurred in the presence or absence of phosphatidylserine and/or calcium, whereas, 1,2-diolein-stimulated protein kinase C activity required both phosphatidylserine and calcium. Dibutyryl cAMP also stimulated kinase activity, particularly in extracts of small cells.

Treatment of large luteal cells with PMA caused a significant shift of protein kinase C activity from the cytosolic to the membrane-bound fraction (Fig. 2). By 15 min after addition of PMA, 91% of the protein kinase C was located in the membrane-bound fraction. Treatment of luteal cells for 12–18 h with 1 μM PMA caused a significant reduction in protein kinase C activity in both large and small luteal cells (93% and 82%,

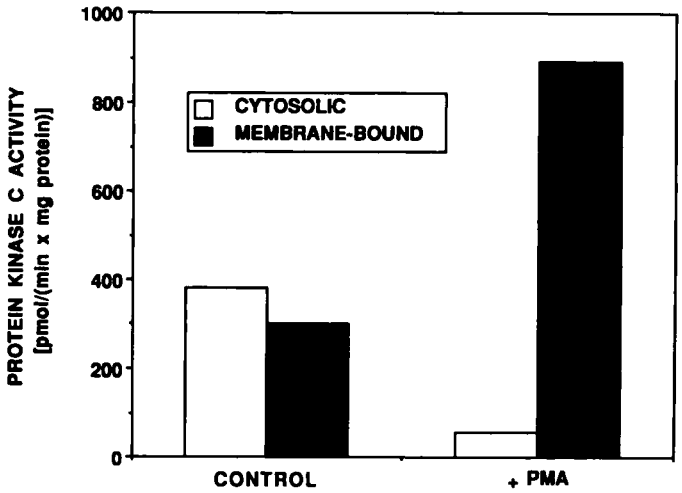


FIG. 2. Effect of treatment with 100 nM phorbol 12- myristate 13-acetate (*PMA*) for 15 min on the distribution of protein kinase C activity in large luteal cells. Cytosolic and membrane-bound protein kinase C activity were measured as described in the *text*. A repeat of this experiment yielded similar results.

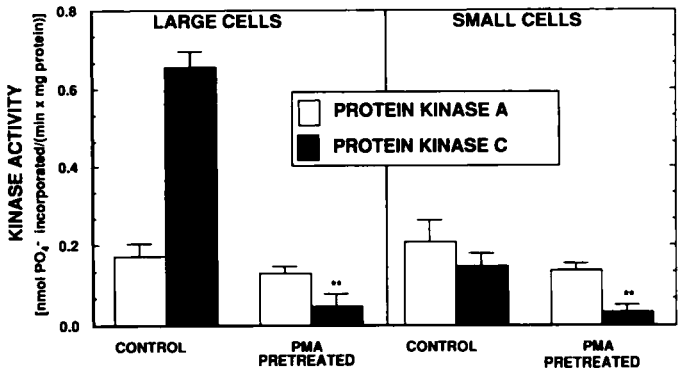


FIG. 3. Effect of 12- to 18-h pretreatment with 1 μM phorbol 12-myristate 13-acetate *(PMA)* or the ethanol vehicle *(Control)* on total protein kinase C or cyclic adenosine 3',5'-monophosphate-dependent protein kinase (protein kinase A) activity in large or small luteal cells (mean ± SEM for 4 experiments). Activity of protein kinase C and A were measured as described in *text*.

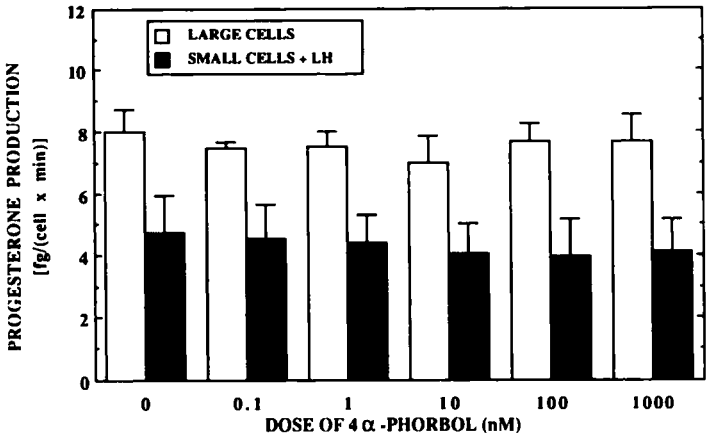


FIG. 5. Lack of an effect of 4α-phorbol, a phorbol ester that does not stimulate protein kinase C, on progesterone production by large and luteinizing hormone *(LH)*-stimulated small luteal cells (mean ± SE for 3 experiments each run in duplicate).

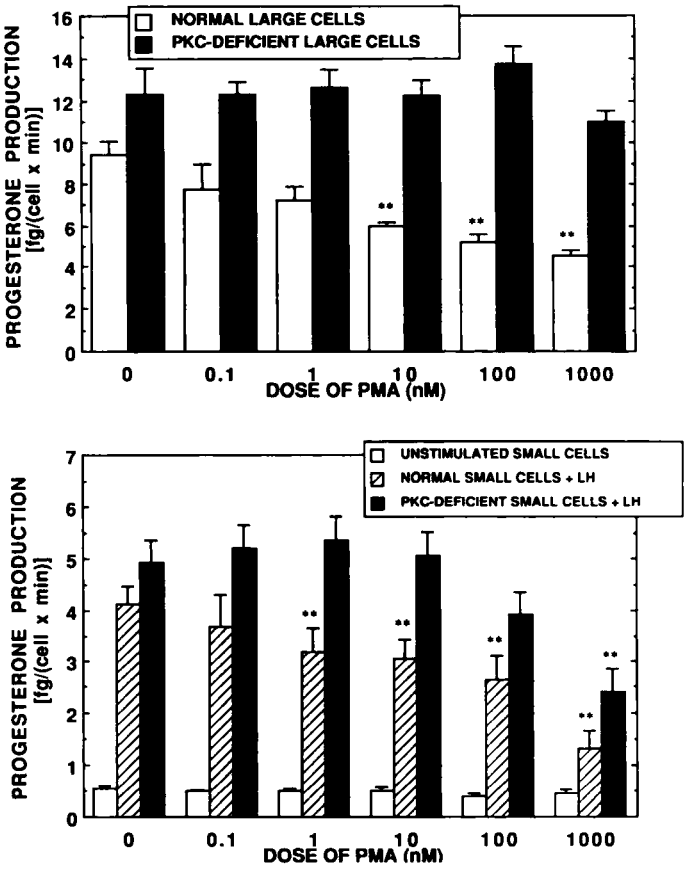


FIG. 4. Dose-dependent effect of acute (2 h) treatment with phorbol 12-myristate 13-acetate *(PMA)* on progesterone production by nonstimulated large luteal cells *(upper graph)* or nonstimulated and luteinizing hormone-stimulated small luteal cells *(lower graph*: mean ± SE for 3 experiments each run in duplicate). *Asterisks* indicate significantly lower than 0 dose of PMA, as determined by analysis of variance followed by Dunnett's *t*-test, $p<0.05$. Protein kinase C-deficient *(PKC-deficient)* cells were produced by preincubating cells for 12–18 h with 1 μM PMA.

respectively), compared to control cells treated with ethanol (Fig. 3). Incubation of large luteal cells for 12 h with 100, 200, or 400 nM PMA also dramatically reduced protein kinase C activity (86%, 91%, and 94%, respectively). Treatment with PMA did not influence protein kinase A activity (Fig. 3), nonspecific protein kinase activity, or cell viability (live cells: normal = 81 ± 3%; PKC-deficient = 78 ± 4%).

Acute (2 h) treatment with PMA caused a dose-dependent decrease in progesterone production by both large luteal cells and LH-stimulated small luteal cells (Fig. 4). However, progesterone production by protein kinase C-deficient large luteal cells or by nonstimulated small luteal cells was not affected by PMA treatment. Progesterone production by protein kinase C-deficient, LH-stimulated small cells was not affected by 0.1–100 nM PMA but was reduced ($p<0.05$) by the 1 μM dose level. 4α-phorbol at concentrations similar to those used for PMA, did not alter progesterone production by either cell type (Fig. 5).

PMA-treated large luteal cells produced significantly less progesterone than nontreated cells by 15 min after treatment ($p<0.01$) and at all subsequent times (Fig. 6A). Progesterone production by LH-stimulated small luteal cells was reduced ($p<0.01$) by PMA by 60 min after treatment (Fig. 6B).

Treatment with PMA had no effect on nonstimulated small cells, but inhibited the production of progesterone ($p<0.01$) by small luteal cells stimulated with LH (1.1 ± 0.2 to 0.6 ± 0.1 fg/cell × min; mean ± SE), forskolin (1.4 ± 0.3 to 0.7 ± 0.1), or dBcAMP (1.3 ± 0.2 to 0.8 ± 0.2) (Fig. 7). Production of pro-

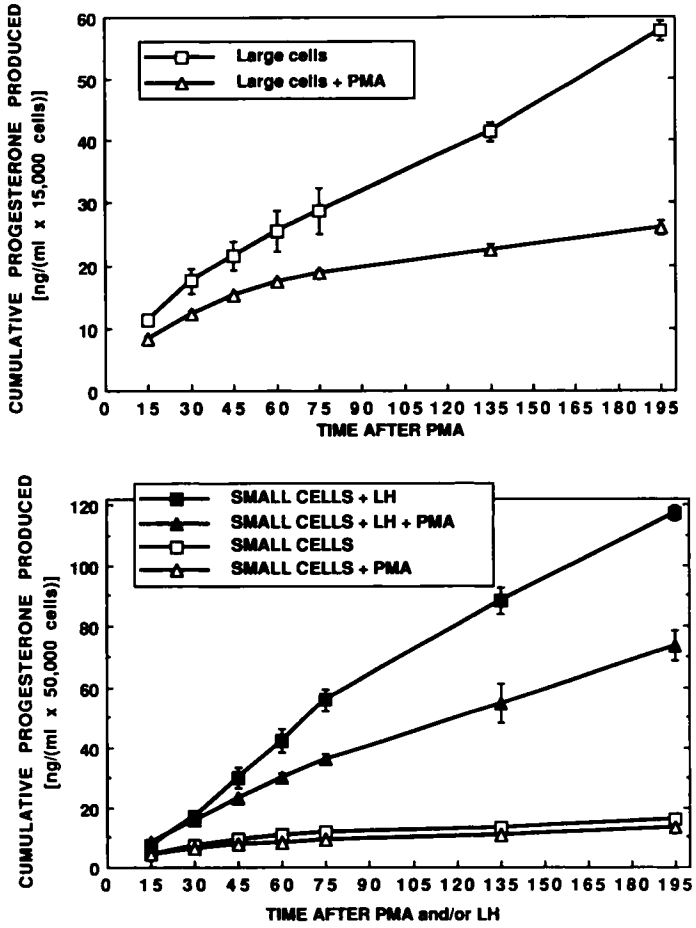


FIG. 6. Representative experiment showing the time-dependent effect of 200 nM phorbol 12-myristate 13-acetate *(PMA)* on progesterone production by nonstimulated large luteal cells *(upper graph)* and nonstimulated or luteinizing hormone *(LH)*-stimulated small cells *(lower graph*: mean ± SD for triplicate wells*)*. At each time point, all medium was removed from cells and replaced with fresh medium containing appropriate treatments.

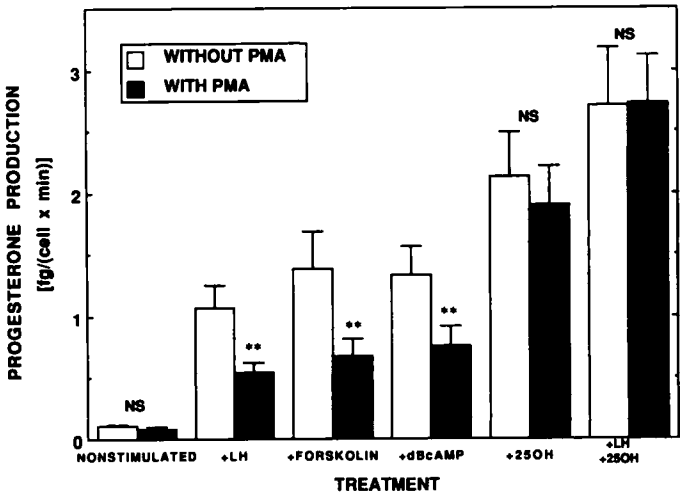


FIG 7. Effect of 200 nM phorbol 12-myristate 13-acetate *(PMA)* on progesterone production by nonstimulated small luteal cells or by small cells treated with 100 ng/ml luteinizing hormone *(LH)*, 50 μM forskolin, 10 mM dibutyryl cyclic adenosine 3′,5′-monophosphate (dBcAMP), or 20 μg/ml 25-hydroxycholesterol *(25OH)* during a 2-h incubation. These doses were chosen because preliminary experiments indicated that they were maximally stimulatory for progesterone production. *Asterisks* indicate that PMA had a significant effect on progesterone production ($p<0.01$): *NS* indicates differences that were not significant statistically. Values represent mean ± SE for 5 experiments, each run in triplicate.

gesterone by small luteal cells treated with 25-hydroxycholesterol was not inhibited by treatment with PMA.

PMA inhibited ($p<0.05$) progesterone production by control (3.2 ± 0.4 to 1.6 ± 0.3) or by 25-hydroxycholesterol-treated (5.6 ± 0.6 to 4.0 ± 0.6) large luteal cells (Fig. 8). This inhibition was not apparent in protein kinase C-deficient large cells (Fig. 8).

## DISCUSSION

Protein kinase C activity has now been found in ovine, porcine, and bovine luteal cells (Davis and Clark, 1983; Noland and Dimino, 1986; Budnik and Mukhopadhyay, 1987; Wheeler and Veldhuis, 1987). In the present study, protein kinase C and protein kinase A activities were measured in purified preparations of

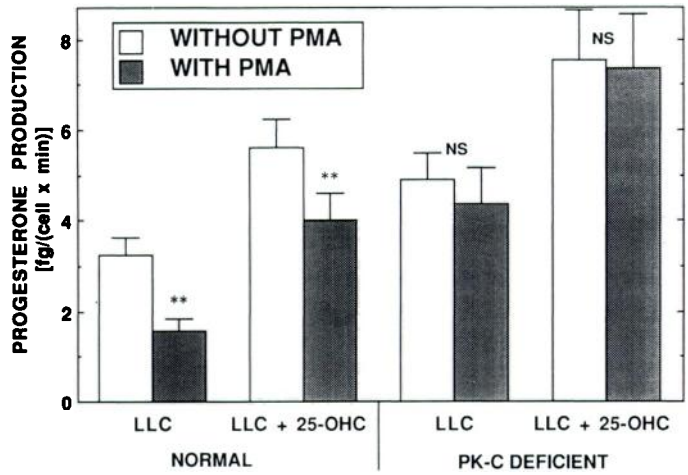


FIG 8. Effect of 200 nM phorbol 12-myristate 13-acetate *(PMA)* on progesterone production by normal or protein kinase C-deficient *(PK-C deficient)* large luteal cells that were either nonstimulated *(LLC)* or treated with 20 μg/ml 25-hydroxycholesterol *(+25-OHC)*. PK-C deficient cells were produced by preincubating cells for 12–18 h with 1 μM PMA. *Asterisks* indicate that PMA had a significant effect on progesterone production ($p<0.05$): *NS* indicates differences that were not significant statistically. Values represent mean ± SE for 5 experiments, each run in triplicate.

large or small ovine luteal cells. Using histone IIIS as the protein substrate, large cells exhibited 4 times greater protein kinase C than protein kinase A activity. Noland and Dimino (1986) reported that pigs have 4 times greater protein kinase C activity in corpora lutea than in mature follicles, but protein kinase A activity was greater in follicles than in corpora lutea (Dimino et al., 1981). As follicular cells differentiate into large luteal cells, they develop a high capacity for progesterone production, which does not appear to be regulated by the stimulatory protein kinase A pathway (Hoyer and Niswender, 1986). Part of this differentiation process may also involve increasing intracellular levels of protein kinase C, since this activity is high in large luteal cells. Therefore, it may be postulated that as large luteal cells differentiate, steroidogenic control switches from primarily stimulatory regulation through the protein kinase A pathway to primarily inhibitory regulation through the protein kinase C pathway.

To evaluate the role of protein kinase C in luteal cells, PMA was used to activate protein kinase C. Translocation of protein kinase C from the cytosolic to the membrane compartments following treatment with PMA was taken as evidence of protein kinase C activation (Anderson et al., 1985; Thomas et al., 1987). By 60 min after PMA-induced translocation of protein kinase C activity to membranes, there was a significant loss of protein kinase C activity, which apparently is due to proteolytic degradation of the enzyme (Wiltbank and Niswender, unpublished; Witters and Blackshear, 1987). By 12 h after treatment with PMA, 93% of protein kinase C in large luteal cells was inactivated. These protein kinase C-deficient cells can be used to evaluate which drug actions or, in subsequent studies, which hormone actions are mediated through protein kinase C.

Addition of PMA caused a rapid and dose-dependent decrease in production of progesterone in both large and LH-stimulated small luteal cells. This effect appeared to be specific for protein kinase C since similar concentrations of 4α-phorbol had no effect, and since protein kinase C-deficient cells showed a greatly attenuated response to PMA. An inhibitory effect of PMA on steroidogenesis has also been reported in rat (Baum and Rosberg, 1987) and sheep (Hoyer et al., 1988) luteal cells and in LH-stimulated small luteal cells from cattle (Benhaim et al., 1987). Paradoxically, it has also been reported that low doses of PMA (Brunswig et al., 1986; Hansel et al., 1987; Alila et al., 1988), particularly during submaximal gonadotropin stimulation (Benhaim et al., 1987), appear to stimulate progesterone secretion from small bovine luteal cells. The lack of a stimulatory effect of PMA in our experiments could be due to use of maximally stimulatory doses of LH or to a difference in the responsiveness of ovine vs. bovine luteal cells. Further, since protein kinase C-deficient cells tended to produce greater quantities of progesterone than normal cells, our results suggest that stimulatory effects of long-term treatment with PMA (Jalkanen et al., 1987) are probably due to elimination of endogenous, inhibitory protein kinase C activity rather than activation of the enzyme.

The inhibitory action of protein kinase C in small luteal cells appears to occur, at least in part, after LH-binding and cAMP production, since forskolin- or dBcAMP-stimulated progesterone production are inhibited similarly to LH-stimulated progesterone production. Benhaim et al. (1987) reported that PMA decreases progesterone production in LH-stimulated small luteal cells of cows with no change in intracellular concentrations of cAMP. It appears from our data that protein kinase C acts prior to the cholesterol side-chain cleavage enzyme in small luteal cells since 25-hydroxycholesterol metabolism was not inhibited by PMA. The metabolism of 25-hydroxycholesterol appears to provide a reasonable estimate of cholesterol side-chain cleavage activity (Toaff et al., 1982). Therefore, protein kinase C probably inhibits cholesterol esterase or cholesterol transport to the side-chain cleavage enzyme in small luteal cells.

In large luteal cells, protein kinase C appears to inhibit cholesterol side-chain cleavage activity since 25-hydroxycholesterol metabolism was inhibited following treatment with PMA. An inhibitory action of protein kinase C prior to cholesterol side-chain cleavage also seems likely since 25-hydroxycholesterol treatment increased progesterone production in PMA-treated large cells. Whether the inhibitory effects of protein kinase C are mediated through direct phosphorylation of steroidogenic enzymes or by some indirect pathway cannot be determined from our data.

In other gonadal tissues, phorbol esters inhibit steroidogenesis by a variety of mechanisms. For example, in bovine granulosa cells, phorbol esters appear to inhibit cholesterol side-chain cleavage (Veldhuis and Demers, 1986). In mouse Leydig cells, phorbol ester inhibits LH-stimulated steroidogenesis by suppressing cAMP production (Mukhopadhyay and Schumacher,

1985). In rat luteal cells, PMA appears to inhibit cAMP production as well as some undefined part of the steroidogenic pathway (Baum and Rosberg, 1987).

At this time it is unclear how luteal protein kinase C is physiologically regulated; however, hormonal activation of this kinase seems likely. $PGF_{2\alpha}$ and LH initiate the breakdown of phosphatidylinositol-4,5-biphosphate to inositol-triphosphate and diacylglycerol in luteal tissue (Davis et al., 1987a,b; Jacobs et al., 1987; Allen et al., 1988), which could provide both the increase in free intracellular calcium and the diacylglycerol that are necessary for activation of protein kinase C. In porcine ovarian cells, treatment with $PGF_{2\alpha}$ causes a translocation of phorbol dibutyrate binding from the cytosolic to the membrane-bound fraction, suggesting that $PGF_{2\alpha}$ activates protein kinase C in ovarian cells (Veldhuis, 1987). Since other second messenger systems are involved in the regulation of luteal steroidogenesis, our use of PMA allowed a more selective activation of the protein kinase C system. The results indicate that this pathway is inhibitory to steroidogenesis in large and small ovine luteal cells. This inhibitory pathway may prove to be of physiologic importance in tonically modulating luteal progesterone production and/or in depressing progesterone production during luteal regression.

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