Protein Kinase C Activity and Its Effect on Progesterone Production by Large and Small Porcine Luteal Cells (44160)

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Abstract. **Protein kinase C (PKC) activity and its effect on progesterone production were investigated using porcine large and small luteal cells (LLC and SLC). Corpora lutea (CL) were surgically collected from pigs on Day 10 of the estrous cycle (Day 0 = onset of standing estrus). Luteal cells were dissociated by collagenase; LLC and SLC were further separated on a discontinuous Ficoll gradient. In a dose-response experiment with phorbol 12-myristate 13-acetate (PMA, a stimulator of PKC), progesterone production was not affected by 0.01 and 0.1 μ*M* PMA, but was stimulated by 1 μ*M* PMA. In a time series experiment, progesterone secretion was increased by 1 and 10 μ*M* PMA in LLC by 60–150 min, and by 1 μ*M* PMA in SLC during 120 and 150 min of incubation. However, 4α-phorbol ester did not affect progesterone synthesis. H-7, a PKC inhibitor, blocked PMA-stimulated progesterone secretion by LLC during 3 hr of incubation.**

Of the PKC activators tested at 10 μ*M*, PMA significantly stimulated cytosolic PKC activity over that of natural PKC activators in both LLC and SLC, whereas 4α-phorbol ester did not affect PKC activity. H-7 inhibited PMA-stimulated PKC activity. PS (1-phosphatidyl-L-serine) + CA^{++} and PS + DG (1,2-dioleoyl-sn-glycerol) + Ca^{++} stimulated PKC activity. The results demonstrate that activation of PKC can increase progesterone secretion by porcine luteal cells from Day 10 of the estrous cycle and suggest PKC can have multiple effects in regulating luteal function. [P.S.E.B.M. 1993, Vol 216]

It is considered that luteinizing hormone (LH) stimulates progesterone production and maintains the corpus luteum (CL) function through protein kinase A (PKA). Conversely, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) inhibits progesterone synthesis and induces degeneration of CL through protein kinase C (PKC) (1). Prostaglandin $F_{2\alpha}$ can significantly decrease *in vivo* or *in vitro* progesterone synthesis and induce luteolysis in ruminants during the middle period (Days 10–12) of the estrous cycle (1). Activators of PKC can directly decrease *in vitro* progesterone secretion by rat luteal cells (2) and ovine large luteal cells (LLC) (3, 4) or inhibit PKA activator-stimulated progesterone synthesis by mixed luteal cells from pregnant hamster (5), rat (6), and mouse (7). It is clear that effects of $PGF_{2\alpha}$ on progesterone production are consistent with the effects of activation of PKC.

Porcine CL is unique from ruminants in that it is significantly refractory to $PGF_{2\alpha}$ *in vivo* over much of the cycle (8, 9). Porcine LLC and small luteal cells (SLC) do not respond to $PGF_{2\alpha}$ *in vitro* (10) and show little responsiveness during the middle period of the estrous cycle (11–13). Protein kinase C activity has been found in luteal tissue from several species (13–15), including pigs (16, 17). However, PKC activity and its effects on progesterone production by different cell types (i.e., LLC or SLC) have not been determined in the pig. This study, therefore, was designed to investigate PKC activity in porcine LLC and SLC and its effects on progesterone production.

Materials and Methods

Preparation of Cells. Cell preparation was basically as described previously (10). In brief, corpora lutea were surgically collected from cyclic gilts on Day 10 of the estrous cycle (first day of standing estrus = Day 0) and

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placed immediately in chilled fresh, sterile media comprised of Ham's F-12 Nutrient Mixture (10.6 g/1 liter; GIBCO, Grand Island, NY) containing 1.176 g of $NaHCO_3$, 0.014 g polymyxin, 0.1 g streptomycin, 0.002 g insulin (Sigma Chemical Co., St. Louis, MO), 0.00004 g hydrocortisone, 0.005 g transferrin (Sigma), and 0.0025 g metronidazole (Sigma) per liter of deionized water. Aseptic techniques were employed throughout and samples were kept on ice or at 4°C unless stated otherwise. The CL were decapsulated, weighed, chopped finely, and placed in 5 ml media/g tissue. Dissociation was carried out using sequential incubation (2 × 45 min; 1 × 30 min) in media containing 2 mg/ml collagenase (Type V, Sigma) in a shaking water bath at 37°C. Pellets formed from centrifugation of the retained supernatant were resuspended in the incubation media containing EGTA (1 m*M*, Sigma) and incubated at 37°C for a further 10 min. Final washing of the resulting cells with media was followed by layering cells onto a discontinuous Ficoll gradient (Pharmacia-LKB, Uppsala, Sweden) at room temperature. After 45 min, Ficoll layers with cells were filtered, pelleted (5 min, 500*g*), and washed four times with fresh media. The cells were counted, viability checked using trypan blue exclusion and incubated overnight at 37°C in 24-well plates (Becton Dickinson Labware, Lincoln Park, NJ) with 10% heat-deactivated bovine calf serum (Sigma).

Experiment 1: Dose Effects of PMA on Progesterone Production by LLC and SLC. The effect of various doses of PMA on progesterone secretion was tested initially. After overnight incubation at 37°C, culture media were removed from culture plates, fresh media (containing 10% heat deactivated bovine calf serum) with or without PMA at 0.0, 0.01, 0.1, or 1.0 μ*M* were added to cells (3×10^4 LLC or 2×10^5 SLC/ml) and the cells were incubated for 2 hr. After 2-hr incubation, media from culture plates were collected and stored at −20°C. Progesterone was determined by radioimmunoassay (RIA). A total of three individual experiments were done with each treatment in triplicate.

Experiment 2: Time Effects of PMA on Progesterone Production by LLC and SLC. From the results of Experiment 1, 1 μ*M* PMA was selected for the time series study. For comparison, 10 μ*M* PMA was also used. Cells were resuspended at the same concentration as Experiment 1, and incubated with PMA or non-PKC activator (4α-phorbol ester) or a PKC inhibitor H-7 (1-[isoquinolinylsulfonyl]-3-methyl piperazine dihydrochloride) (18), with 10% heat-deactivated bovine calf serum/ml in 24-well plates. Media in the culture plates were collected at 20- or 30-min intervals during 3-hr incubation. Levels of treatment were tested in triplicate for luteal cells from each pig. A total of four pigs were used in this experiment.

Experiment 3: Measurement of PKC Activity. In view of the results in Experiments 1 and 2 implicating PKC involvement in progesterone production, this experiment was designed to measure PKC activity under comparable treatment conditions. In order to have sufficient cells for the assay of PKC activity, corpora lutea from the ovaries of two gilts at Day 10 of the estrous cycle were used in each assay. This was repeated four times ($n = 8$ pigs). The cells were resuspended at concentrations of 2×10^6 LLC or 2×10^6 SLC/ml of incubation media with 10% heat-deactivated calf serum, placed in 28.6 × 104–mm sterile polycarbonate tubes (Nalge Company, Rochester, NY) and incubated at 37°C (95% air/5% CO_2/100% humidity) overnight. Following overnight incubation, cells were washed twice with the media and used for the PKC assay.

Protein kinase C activity was determined using Amersham's PKC assay system (Code RPN 77, Amersham, Arlington Heights, IL), which is similar to other methods for PKC measurement (3, 17). However, this PKC assay system contained a peptide substrate (eight amino acids, $M_r = 1098$) with greater specificity for PKC and less for cAMP-dependent protein kinase than the histone IIIS commonly used (Amersham, Code RPN 77).

The enzyme assay is based on the PKC catalytic transfer of the γ-phosphate group of [^{32}P]ATP to the peptide. In preparation for the assay, the cells were centrifuged (500*g*), washed twice using fresh media, and then transferred to numbered polystyrene tubes (12 × 75 mm) with 2-ml ice-cold buffer (pH 7.5), which contained 10 m*M* Tris (hydroxy methyl) amino methane (Sigma), 5 m*M* EDTA, 10 n*M* EGTA, 10 n*M* benzamidine, 0.1% w/v β-mercaptoethanol, 50 μg/mol PMSF (Sigma) and 100 μl leupeptine (Sigma). To determine cytosolic PKC activity, cells in the buffer were sonicated for 15 sec (Sonic Demembrator, Model 300; Fisher). The cell lysate was centrifuged at 16,000*g* for 15 min at 4°C in a microcentrifuge (EC CENTRA-M, International Equipment Company, U.S.A.). The supernatant (termed cytosol) was then separated from the lysate, and 25 μl was used for cytosolic PKC assay (see below). The PKC activity in the cytosol was termed cytosolic PKC (2). To determine total PKC activity, the cell lysate was first incubated with the buffer containing 0.2% Triton X-100 (Sigma) for 45 min to solubilize the membrane-bound PKC (3, 17) and then centrifuged at 16,000*g* for 15 min at 4°C in the microcentrifuge. The PKC activity obtained in this way was termed total PKC activity (cytosol + membrane-bound).

All assay components were brought to 25°C immediately prior to beginning the assay. The reaction mixture (total 75 μl) included 25 μl supernatant, 25 μl magnesium [γ-^{32}P]ATP (2 mCi/ml, Code PB 168, Amersham), and 25 μl component mixture (Amersham RPN 77), consisting of 900 μ*M* peptide, 30 m*M* dithiothreitol (DTT), 45 m*M* magnesium/150 μ*M* ATP. The component mixture also contained either (a) no extra compounds (basal PKC activity) or (b) putative activators of PKC: 10 μ*M* PMA, 10 μ*M* DG (1,2-dioleoyl-sn-glycerol), and 10 μ*M* PS (1-phosphatidyl-L-serine), or (c) the inhibitor H-7 (10 μ*M*) or (d) 4α-phorbol ester (10 μ*M*, Sigma) or dbcAMP (dibutyryl cAMP, 10 μ*M*; Sigma). The reaction was carried out at 25°C in a water bath for 15 min and terminated by addition of 100 μl of a diluted acid (stop reagent, Amersham RPN 77). Thereafter, 125 μl

of the aliquot was removed and placed on numbered binding papers. The papers were individually washed using 5% v/v acetic acid and then placed into individual scintillation vials and counted using a liquid scintillation counter (Wallac Oy, Helsinki, Finland). All test levels were performed in triplicate. Nonspecific binding was checked in each assay.

Measurement of Progesterone. The concentration of progesterone was determined by RIA without ether extraction (10) using progesterone antisera raised in rabbits (A18, N. Rawlings, University of Saskatchewan). The inter- and intraassay coefficients of variation were 9% and 6%, respectively. Progesterone was expressed as ng progesterone per 2×10^5 (SLC) or per 3×10^4 (LLC) cells. The mean sensitivity of the assay at 95% binding was 12.5 pg/tube.

Statistical Analysis. For Experiment 1, 2, and 3, the compounds were used in a completely randomized block design. Blocks consisted of individual pigs on Day 10. The error used to test treatment effects was the treatment by block interaction term. Data were analyzed by the General Linear Models Procedure (19). Treatment effects were evaluated for each cell type. Differences were tested using the Bonferroni test (19). Values were presented as LSM ± SEM.

Protein Determinations. Protein determinations were made by the Bio-Rad method (Richmond, CA) utilizing BSA as a standard.

Results

Cell viability, as determined by 0.4% trypan blue exclusion, was between 80% and 85% after isolation. After 2–3 hr of incubation with or without PMA or other treatments, cell viability was between 71% and 82%. PMA treatment did not influence cell viability. Contamination of the SLC by the LLC was 0.27% ± 0.11% (mean ± SEM) and the contamination of the LLC by the SLC was 18% ± 1.84% (mean ± SEM).

Experiment 1: Dose Effect of PMA on Progesterone Synthesis by LLC and SLC. Progesterone secretion by LLC and SLC was not affected by 0.01 and 0.1 μM PMA but was increased ($P < 0.05$) by 1 μM PMA after 2 hr of incubation (Fig. 1, A and B).

Experiment 2: Time Effect of PMA on Progesterone Synthesis by LLC and SLC. Frequent samples of media collected during 3 hr of incubation showed that both 1 and 10 μM PMA increased progesterone production by LLC within 180 min (Fig. 2A). Increased progesterone levels were noted between 60 and 150 min of incubation with 1 μM PMA and at 120 min with 10 μM PMA ($P < 0.05$), respectively. In the SLC, 1 μM but not 10 μM PMA increased progesterone production by 150 and 180 min ($P < 0.05$, Fig. 2B).

Because the stimulatory effect of PMA by porcine LLC in these experiments was different from the results with ruminants (3) at a similar period during the estrous cycle, the PKC inhibitor H-7 was used to test if it could inhibit PMA's stimulatory effects on progesterone production by

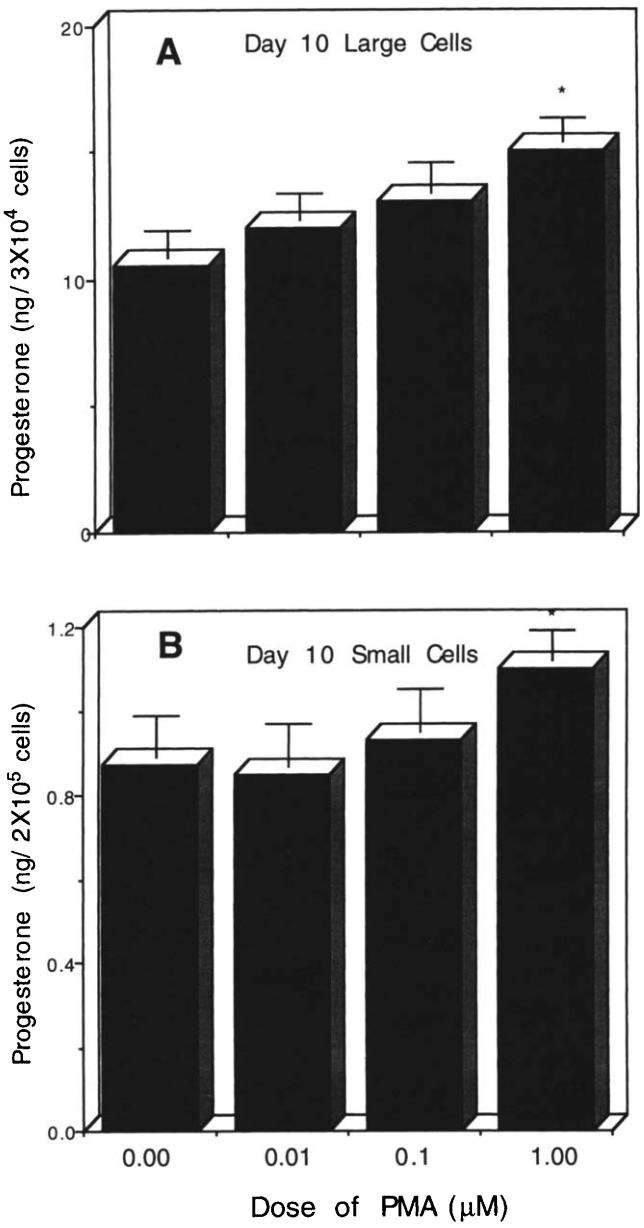

Figure 1. The effect of different doses of PMA (0, 0.01, 0.1, 1 μM) on progesterone production (ng/3 × 10^4 large cells and ng/2 × 10^5 small cells, LSM ± SEM) after 2 hr of incubation in large (A) and small (B) luteal cells on Day 10 during the estrous cycle ($n = 4$ pigs). *$P <$ 0.05, different from control level (0 μM).

LLC. Ten micromolar PMA increased progesterone secretion, similar to that seen in Figure 2. H-7 alone did not affect progesterone synthesis but inhibited PMA-stimulated progesterone secretion by LLC (Fig. 3). 4α-Phorbol ester was without effect.

Experiment 3: Protein Kinase C Activity in LLC and SLC. Since 10 μM PMA consistently showed stimulatory effects, especially by LLC (Figs. 2 and 3), the same dose of PMA was used for this experiment. Other natural PKC activators (10 μM) were also used. PKC activity was expressed as pmole/min/mg protein (LSM ± SEM).

Basal cytosolic PKC activity in the LLC (only cytosol and the peptide substrate were present in the reaction mix-

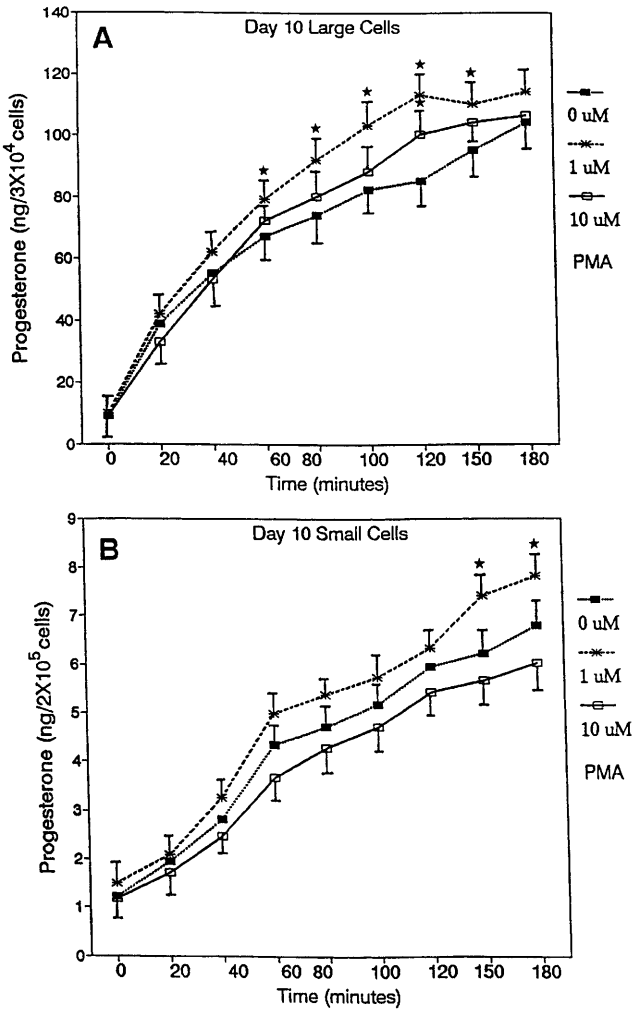

Figure 2. The effect of different doses of PMA (0, 1, 10 μ*M*) on progesterone production (ng/3 x 10^4 large cells and ng/2 x 10^5 small cells, LSM ± SEM) at 20- or 30-min intervals within the first 3 hr of incubation in the large (A) and small (B) luteal cells on Day 10 during the estrous cycle (n = 4 pigs). *P < 0.05, different from control level (0 μ*M* PMA).

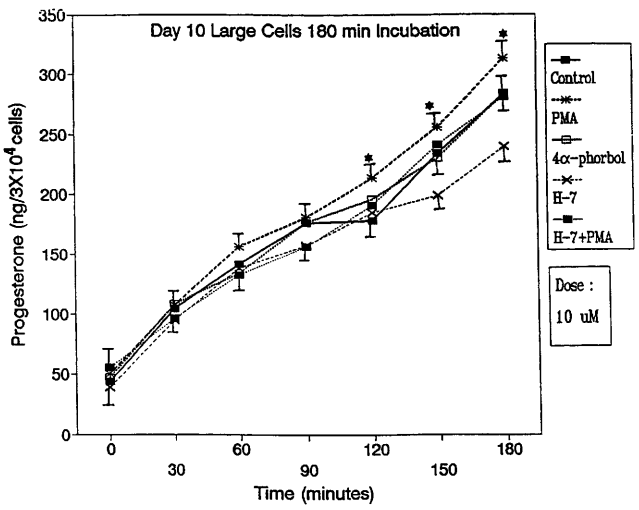

Figure 3. The effect of 10 μ*M* PMA, 4α-phorbol ester, H-7 or H-7 + PMA on progesterone production in large luteal cells on day 10 (ng/3 x 10^4 cells, LSM ± SEM, n = 6 pigs). *P < 0.05, different from control.

ture without any added PKC activator) was 3-fold greater than in SLC (Fig. 4, A and B). In LLC, total PMA-stimulated PKC activity was about 2-fold greater than in cytosol (not shown). Of the PKC activators tested, PMA had the greatest effect in both LLC and SLC. Cytosolic PKC activity in both cell types was not influenced by nonactivators of PKC: 4α-phorbol ester; dbcAMP; Ca^{++}; and DG + Ca^{++}. But PS + Ca^{++} and PS + DG + Ca^{++} stimulated PKC activity (Fig. 4). H-7 inhibited PMA-stimulated cytosolic PKC activation in both LLC and SLC (Fig. 5).

Discussion

Porcine LLC are larger than SLC and secrete more progesterone. Unlike in the sheep and cow, porcine LLC contain large fingerprint smooth endoplasmic reticulum (SER) (10). Phorbol 12-myristate 13-acetate is a well-known PKC activator and widely used for studying PKC activity (17, 20) and steroidogenesis in luteal cells (1). PMA-stimulated PKC activity in membrane and cytosol in LLC in the present study is similar to that in the ewe (3), indicating a similar PKC distribution in these two species. Furthermore, PMA significantly stimulated cytosolic PKC activity and H-7, the PKC inhibitor (18), significantly inhibited PMA-stimulated PKC activity. Corresponding to PKC activity, PMA increased progesterone secretion by LLC and SLC during 3 hr of incubation, whereas H-7 inhibited PMA-stimulated progesterone secretion. These results strongly suggest that activation of PKC by PMA increases progesterone secretion by porcine luteal cells. This is notably different from ruminants, where activation of PKC inhibits progesterone production by LLC around Day 10–12 of the estrous cycle (3, 21). On the other hand, low concentrations of PMA (10 and 100 n*M*) did not induce any change in progesterone secretion. Since phorbol esters can insert themselves into the plasma membrane and are disposed of very slowly (22, 23), these lower concentrations may not have been sufficient to reach a critical level for PKC activated influence on progesterone production.

The stimulatory effect of activation of PKC on progesterone secretion is also found in other studies. For example, activation of PKC stimulates *in vitro* progesterone synthesis by bovine SLC around Day 10 of the estrous cycle (24–26), by porcine luteal tissue (27), and by human granulosa-lutein cells (28). It is not clear how activation of PKC causes this reaction. However, recent studies of cross-talk between PKC and PKA systems indicate that endogenous cAMP activation of PKC (29, 30) may explain the reaction. Pretreatment with PMA increased the response of adenylate cyclase to a subsequent hormonal stimulation without changing the affinity of the receptors for the hormone in bovine luteal cells (31). In mixed swine luteal cells, PMA increased LH- and forskolin-activated cAMP accumulation. DG mimicked the effect of PMA (32). The cAMP accumulation elicited either by receptor activation or by the activation of adenylate cyclase will induce progesterone pro-

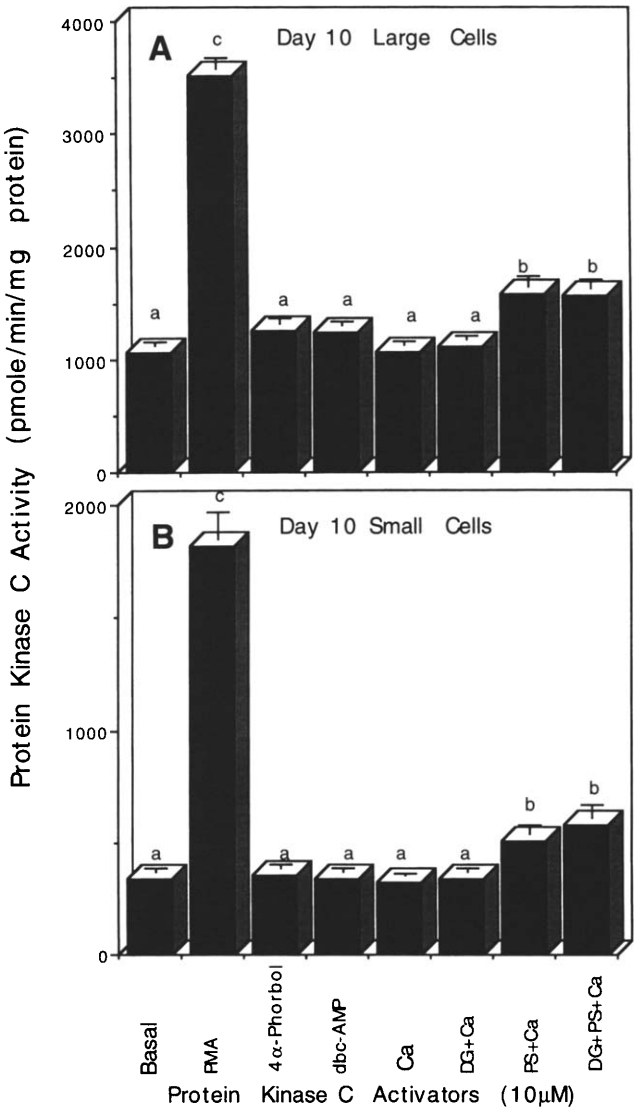

Figure 4. Influence of PMA and natural PKC activators on PKC activity (pmole/min/mg protein, LSM ± SEM) in large (A) or small (B) luteal cells (n = 8 pigs) on Day 10. Columns that do not have a common letter differ significantly ($P < 0.05$).

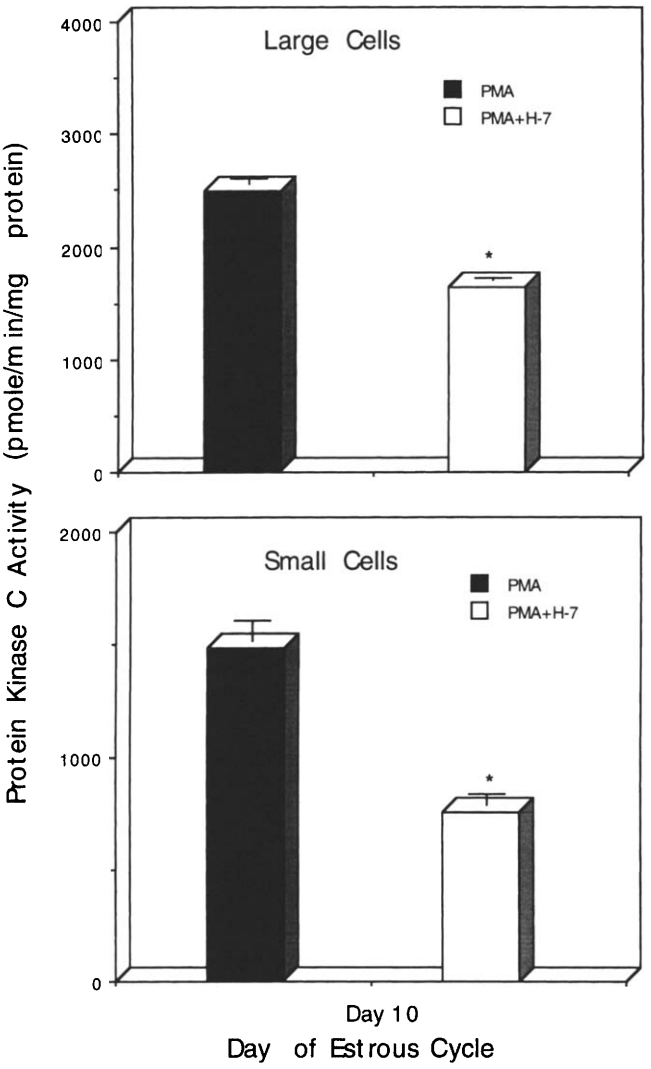

Figure 5. Effects of H-7 on PMA-stimulated PKC activity (pmole/min/mg protein, LSM ± SEM) in large or small luteal cells (n = 8 pigs) on Day 10. *$P < 0.05$.

duction by luteal cells (1). Although the present study did not determine cAMP production and its related hormonal stimulation on progesterone production, stimulatory effects by cAMP have been found in luteal cells *in vitro* (5–7). Cyclic AMP could be responsible for increased progesterone secretion in PMA-treated LLC.

The protein kinase C family consists of at least 10 isozymes which display multiple effects (both stimulatory or inhibitory) in different tissues or cells (33). Protein kinase C is normally cleared by the neutral protease (calpain I and II) to produce catalytically active fragments (isozymes). Physiologically, calpain is not only responsible for producing a catalytically active fragment of PKC (34), but also for proteolytic degradation of the enzyme (35). In PKC family, γ subspecies (isozyme) is very susceptible to calpain, whereas α subspecies is relatively resistant to proteolysis (35). In nonluteal tissues, effects of activation of PKC depend on its isozymes. For example, activation of PKC enhances the responses of cAMP-generating systems in PC12 cells, which contain both α and γ isozymes of PKC. In NCB20 cells and NIH 3T3 cells, where only the α subspecies is expressed, activation of PKC causes inhibition of cAMP-generating systems. In NIH 3T3 cells after transfection of γ-PKC, activation of the enzyme is no longer inhibitory; instead, there is a facilitation of cAMP accumulation (30). Six PKC isoforms have been found in bovine luteal cells (36), but their individual effects on steroidogenesis have not been identified. It is possible that PKC isozymes could result in its multiple reactions in luteal cells (i.e., stimulatory [24–27, 29] or inhibitory [3–7, 21] to progesterone production). The nature of the response depends on species, cell type (LLC or SLC), collecting time of CL, and doses of activators of PKC used in each experiment.

Porcine CL are well known for their *in vivo* refractoriness to $PGF_{2\alpha}$ during the midcycle. Injection of $PGF_{2\alpha}$ cannot readily induce luteolysis until Day 12 of the estrous

cycle in the pig (8, 9). *In vitro,* $PGF_{2\alpha}$ did not inhibit progesterone synthesis by LLC on Day 10 (10). However, $PGF_{2\alpha}$ can induce significant luteolysis *in vivo* or inhibit progesterone production *in vitro* by LLC in ruminants (1). As far as we know, no extant studies can clearly explain this species discrepancy. Our results might provide a clue suggesting that the refractoriness of porcine CL to $PGF_{2\alpha}$ could be due to its PKC activity—that is, PKC could not transfer the $PGF_{2\alpha}$ inhibitory signals to cells during the middle period of the estrous cycle.

Although PMA stimulated PKC activity in SLC, the response in terms of progesterone secretion differed from LLC in time and magnitude. In the sheep, PKC activity was increased to 2.9-fold in SLC by PKC activators, but PMA alone did not affect progesterone production in SLC and only decreased LH-stimulated progesterone synthesis (3). The reason for this different response to PMA between LLC and SLC are unknown. Low PKC activity observed in SLC may be one reason.

Natural activators of PKC in cells are PS, DG, and Ca^{++}. They did not individually activate PKC, and only combination of PS + Ca^{++} and PS + DG + Ca^{++} increased PKC activity. Because cells normally contain PS, DG, and Ca^{++}, they may have already activated PKC in luteal cells prior to treatment with DG, PS, or Ca^{++}. The activation by PS + Ca^{++} and PS + DG + Ca^{++} supports the requirement of PKC for PS, DG, and Ca^{++} for its activity even though their effects were less pronounced than with PMA.

In summary, PKC activity was present in porcine LLC and SLC, and PMA increased progesterone production, particularly by LLC. These results suggest that activation of PKC increases progesterone production and that PKC has multiple effects in regulating luteal function. These results also suggest that the refractoriness of porcine CL to $PGF_{2\alpha}$ around Day 10 of the estrous cycle may be due to the stimulatory activity of PKC in steroidogenesis, which could prevent the transfer of a $PGF_{2\alpha}$ inhibitory signal in luteal cells.

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1. Niswender GD, Nett TM. The corpus luteum and its control in infra primate species. In: Knobil E, Neill IJ, Eds. The Physiology of Reproduction. New York: Raven Press, pp781–816, 1994.
2. Baum MS, Rosberg S. A phorbol ester, phorbol 12-myristate 13-acetate, and a calcium ionophore, A23187, can mimic the luteolytic effect of prostaglandin F2α in isolated rat luteal cells. Endocrinology **120:**1019–1026, 1987.
3. Wiltbank MC, Knickerboker JJ, Niswender GD. Regulation of the corpus luteum by protein kinase C. I. phosphorylation activity and steroidogenic action in large and small ovine luteal cells. Biol Reprod **40:**1194–1200, 1980.
4. Wiltbank MC, Diskin MG, Flores JA, Niswender GD. Regulation of the corpus luteum by protein kinase C. II. Inhibition of lipoprotein stimulated steroidogenesis by prostaglandin $F_{2\alpha}$. Biol Reprod **42:**239–245, 1990.
5. Yuan W, Greenwald GS. Luteotropic effects of follicle stimulating hormone (FSH): II. FSH, luteinizing hormone and prolactin effects on second messenger systems in the corpus luteum of the pregnant hamster. Biol Reprod **51:**472–479, 1994.
6. Yuan W, Greenwald GS. *In vitro* effects of FSH, LH and prolactin on progesterone synthesis by rat luteal cells during pregnancy. Proc Soc Exp Biol Med **209:**376–381, 1995.
7. Yuan W, Greenwald GS. Progesterone production *in vitro* by mouse luteal cells: Response to FSH, LH, and prolactin. Proc Soc Exp Biol Med **214:**265–270, 1996.
8. Connor ML, Phillips GD, Palmer WM. Effect of prostaglandin $F_{2\alpha}$ on the estrous cycle and hormone levels in the gilt. Can J Anim Sci **56:**661–669, 1976.
9. Britt JH. Induction and synchronization of ovulation. In: Hafez ES, Ed. Reproduction in Farm Animals (5th ed.). Philadelphia: Lea & Febiger, pp507–527, 1987.
10. Yuan W, Connor ML, Buhr MM. Responsiveness of porcine large and small luteal cells to luteotropic or luteolytic hormones and cell morphologic changes during the estrous cycle and pregnancy. J Anim Sci **71:**481–491, 1993.
11. Hunter MG. Responsiveness *in vitro* of porcine luteal tissue recovered at two stages of the luteal phase. J Reprod Fertil **63:**471–476, 1981.
12. Buhr MM. Effect of lipoproteins and luteinizing hormone on progesterone production by large and small luteal cells throughout the porcine estrous cycle. J Anim Sci **65:**1027–1033, 1987.
13. Agu GO, Buhr MM. Progesterone production *in vitro* by luteal cells from hormonally-induced prepubertal gilts. Can J Anim Sci **70:**987–990, 1990.
14. Davis JS, Clark MR. Activation of protein kinase in the bovine corpus luteum by phospholipid and Ca^{2+}. Biochem J **214:**569–574, 1983.
15. Clark MR, Davis JS, Lemaire WJ. Calcium- and lipid-dependent protein phosphorylation in the human ovary. J Clin Endocrinol Metab **57:**872–874, 1983.
16. Noland TA Jr., Dimino JJ. Characterization and distribution of protein kinase C in ovarian tissue. Biol Reprod **35:**863–872, 1986.
17. Wheeler MB, Veldhuis JD. Catalytic and receptor-binding properties of the calcium-sensitive phospholipid-dependent protein kinase (protein kinase C) in swine luteal cytosol. Mol Cell Endocrinol **50:**123–129, 1987.
18. Goodman HM, Tai LR, Chipkin SR. The isoquinoline sulfonamide inhibitor of protein phosphorylation, H-7, H-8, and HA-1004, also inhibit RNA synthesis: Studies on responses of adipose tissue to growth hormone. Endocrinology **126:**441–445, 1990.
19. SAS. SAS User's Guide. Cary, NC: SAS Institute Inc., 1985.
20. Kraft AS, Anderson WB. Phorbol esters increase the amount of Ca^{++} phospholipid-dependent protein kinase associated with plasma membrane. Nature **312:**621–623, 1983.
21. Wiltbank MS, Diskin MG, Niswender GD. Differential actions of second messenger systems in the corpus luteum. J Reprod Fertil **43**(Suppl):65–75, 1991.
22. Michell RN. Ca^{2+} and protein kinase C: Two synergistic cellular signals. Biochem Sci **8:**263–265, 1983.
23. Berridge MJ. Rapid accumulation of inositol triphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem J **212:**849–858, 1983.
24. Hansel W, Dowd JP. New concepts of the control of corpus luteum function. J Reprod Fertil **78:**755–768, 1986.
25. Alila MW, Doud JP, Corradino RA, Harris WV, Hansel W. Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. J Reprod Fertil **82:**645–655, 1988.
26. Brunswig B, Mukhopadhyay AK, Budnik LT, Bohnet HG, Leidenberge FA. Phorbol ester stimulates progesterone production by isolated bovine luteal cells. Endocrinology **118:**743–749, 1986.
27. Veldhuis JD. Prostaglandin $F_{2\alpha}$ initiates polyphosphatidylinositol hy-

drolysis and membrance translocation of protein kinase C in swine ovarian cells. Biochem Biophys Res Commun **149:**112–117, 1987.
28. Jalkaner J, Ritvos O, Huhtaniemi I, Stenman U-H, Laatikainen T, Ranta T. Phorbol ester stimulates human granulosa-luteal cell cyclic adenosine 3′,5′-monophosphate and progesterone production. Mol Cell Endocrinol **51:**273–276, 1986.
29. Bell RM. Protein kinase C activation by diacylglycerol second messengers. Cell **45:**631–632, 1987.
30. Gusovsky F, Gutkind JS. Selective effects of protein kinase C isozyme on cyclic AMP accumulation. Mol Pharmacol **39:**124–129, 1991.
31. Budnik LT, Mukhopadhyay AK. Desensitization of LH-stimulated cyclic AMP accumulation in isolated bovine luteal cells-effect of phorbol ester. Mol Cell Endocrinol **54:**51–61, 1987.
32. Wheeler MB, Veldhuis JD. Facilitative actions of the protein kinase C effector system on hormonally stimulated adenosine 3′,5′-monophosphate production by swine luteal cells. Endocrinology **125:**2414–2420, 1989.
33. Asaoka Y, Nakamura SI, Yoshida K, Nishizuka Y. Protein kinase calcium and phospholipid degradation. Trends Biochem Sci **17:**414–417, 1992.
34. Mikawa K. Studies on proteolysis of protein kinase C with calpain I and II. Kobe J Med Sci **36:**55–69, 1990.
35. Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka M, Kuroda T, Nishizuka Y. Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem **264:**4088–4092, 1989.
36. Khan SA, Joslyn M, Westfall SD, Davis JS. Characterization of protein kinase C (PKC) isoforms in bovine corpus luteum. Biol Reprod **50**(Suppl 1):89(Abstract 139), 1994.