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Regulation of Cytochrome $P450_{scc}$ Synthesis and Activity in the Ovine Corpus Luteum

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The rate-limiting step in luteal biosynthesis of progesterone consists of cleavage of the side chain of cholesterol by mitochondrial cytochrome P450 side-chain cleavage enzyme ($P450_{scc}$) to form pregnenolone. Luteal mRNA encoding $P450_{scc}$, quantitated on selected days of the 16-day ovine estrous cycle, was similar on days 3 and 6, increased by 2-fold on day 9 ($P < 0.05$) and remained elevated on day 15. Levels of $P450_{scc}$ mRNA on day 15 of pregnancy were not different from those found on any day of the cycle ($P < 0.05$). To determine whether levels of mRNA encoding $P450_{scc}$ are hormonally regulated, ewes on day 10 of the estrous cycle were injected with hCG or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). $P450_{scc}$ mRNA was not increased for up to 36 h after injection of hCG, nor decreased within 8 h after injection of $PGF_{2\alpha}$ ($P < 0.05$). An assay for $P450_{scc}$ activity was developed which utilized ovine small and large luteal cells in the presence of 22R-hydroxycholesterol and ovine high density lipoprotein. Enzyme activity was quantitated by measurement of progesterone production. In small luteal cells activation of the protein kinase A (PKA) second-messenger system by treatment with LH resulted in 910% increase in progesterone production without altering activity of $P450_{scc}$. Activation of the protein kinase C (PKC) second-messenger system with phorbol 12-myristate 13-acetate caused a 51% reduction in progesterone secretion from large luteal cells but did not alter activity of $P450_{scc}$. These findings suggest that in mature luteal tissue steady state levels of mRNA encoding $P450_{scc}$, and enzyme activity are independent of acute regulation by activation of PKA or PKC second-messenger systems.

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INTRODUCTION

In sheep, as in other mammals, estrous cycles of normal duration and maintenance of pregnancy depend upon secretion of progesterone by the corpus luteum. The rate-limiting step in this process is conversion of cholesterol to pregnenolone by mitochondrial cytochrome P450 side-chain cleavage enzyme ($P450_{scc}$) [1]. In several species cholesterol side-chain cleavage appears to be regulated by LH, which stimulates synthesis of enzyme [2–4] and mRNA encoding the enzyme [5, 6] in granulosa cells. Once luteinization has occurred, $P450_{scc}$ is thought to be constitutively produced and thus less dependent upon hormonal regulation [7]. The level of $P450_{scc}$ enzyme is low in follicles of the cow [8] and rat [4], increases in the corpus luteum, and decreases as the corpus luteum regresses. Enzyme concentrations are paralleled by levels of mRNA encoding $P450_{scc}$ in bovine and human granulosa and luteal cells [9, 10].

Regulation of luteal $P450_{scc}$ activity by protein kinase A (PKA) and C (PKC) has been investigated utilizing exogenous hydroxylated substrates. Increased conversion of 25-hydroxycholesterol to progesterone and pregnenolone by porcine granulosa cell mitochondria following lengthy pretreatment with FSH and estradiol was correlated with increased enzyme mass [2]. Wiltbank *et al.* [11] assessed side-chain cleavage activity in ovine luteal cells using ovine high density lipoprotein (oHDL) to facilitate uptake of hydroxycholesterols; progesterone production was unaffected by activation of PKA or PKC. Similar studies conducted without lipoprotein supplementation have yielded conflicting

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results [12–14]. A preliminary goal of this study was to establish a kinetically valid *in vitro* assay for cholesterol side-chain cleavage activity over a range of time and tissue concentrations. Since activation of PKA increases production of progesterone in ovine small steroidogenic luteal cells, and PKC activation decreases progesterone production in ovine large steroidogenic luteal cells, partially purified preparations of ovine luteal cells offer a useful system in which the effects of PKA and PKC activation upon activity of cytochrome $P450_{scc}$ might be clarified.

No data exist regarding luteal content of $P450_{scc}$ enzyme or mRNA throughout the estrous cycle of sheep. However, loss of luteal steroidogenic activity during luteolysis in sheep is initiated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) through activation of the PKC second-messenger system [15]. Pharmacologic activation of PKC with phorbol 12-myristate 13-acetate (PMA) also inhibits PKA-stimulated increases in mRNA encoding $P450_{scc}$ in rat granulosa cells [16].

The current study was designed to quantitate luteal mRNA encoding $P450_{scc}$ throughout the reproductive cycle of the sheep. Additionally, experiments were designed to characterize the effect of *in vivo* treatment with hCG and $PGF_{2\alpha}$ upon luteal levels of $P450_{scc}$ mRNA

EXPERIMENTAL

Materials

The cDNA probe complementary to mRNA which encodes bovine adrenal $P450_{scc}$ [17] was provided by Dr M. R. Waterman (Vanderbilt University, Nashville, TN). Medium 199 (M199), restriction enzymes *Hae*III and *Hind*III, and ϕx DNA were obtained from Gibco (Grand Island, NY), and restriction enzyme *Pvu*II from Promega (Madison, WI). Hybond nylon filters and [α^{32}P]dCPT were purchased from Amersham (Arlington Heights, IL). Corning 24-well plastic culture plates were purchased from VWR (Denver, CO). Sodium acetate, sodium chloride, sodium dodecyl sulfate (SDS), deoxyribonuclease I (DNase), hCG, penicillin G, streptomycin sulfate, PMA, cholesterol, 20α-hydroxycholesterol, 22R-hydroxycholesterol, 25-hydroxycholesterol and dimethyl sulfoxide (DMSO) were obtained from Sigma (St Louis, MO). Collagenase D was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Lutalyse was obtained from Upjohn Corp. (Kalamazoo, MI) and LH (NIDDK-oLH-25) from the National Hormone and Pituitary Program (Bethesda, MD). HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was purchased from United States Biochemical Corp. (Cleveland, OH). Agarose Bio-Gel A-5m and Biorad protein reagent were purchased from Biorad (Richmond, CA). Falcon 96-well plastic microtiter plates were obtained from Baxter Diagnostics, Inc. (McGaw Park, IL).

Northern blot procedures

Specificity of hybridization of the labeled cDNA probe was demonstrated and sample RNA analyzed using Northern blot analysis. Total RNA was extracted from 100–300 mg of tissue by the method of Chomczynski and Sacchi [18]. The 1.5 kb cDNA insert was isolated from plasmid DNA pcD, Okayama fragment [19] by restriction digestion with *Pvu*II. Radiolabeled cDNA transcripts of $P450_{scc}$ and tubulin were synthesized using random primer extension to incorporate [α^{32}P]dCTP [20]. Twenty or 25 μg of total RNA isolated from steroidogenic (adrenal and corpus luteum) and non-steroidogenic tissue (liver, uterus, brain, kidney and cardiac and skeletal muscle) were loaded onto 1.5% agarose gel containing 3% formaldehyde and electrophoresed for 1.5 h at 250 V. Radiolabeled ϕx phage DNA digested with *Hae*III or λ phage DNA digested with *Hind*III endonuclease were used as molecular weight markers. RNA was passively transferred to nylon filters and subjected to ultraviolet crosslinking (Stratagene, La Jolla, CA). Filters were hybridized for 36–48 h at 65°C in 10 ml buffer containing approx. 10^7 cpm of radiolabeled cDNA and 150 mg denatured sperm DNA. The filters were washed three times with gentle agitation at room temperature and once at 65°C, in buffer consisting of SSC (0.3 M sodium chloride, 0.03 M sodium citrate at pH 7.0) and 0.1% SDS. Exposure of the hybridized filters to Kodak X-Omat film at −70°C for 24–48 h produced autoradiograms which were quantitated by scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA; Model GS300) [21]. Specific $P450_{scc}$ mRNA was quantitated relative to tubulin mRNA to reduce variation caused by unequal loading of the gels. Data, expressed as relative densitometric units, were analyzed by analysis of variance and Duncan's multiple range test.

Serum levels of progesterone were determined by radioimmunoassay [22]; tissue concentrations of progesterone were assessed by the methods of Diekman *et al.* [23]. Intra- and interassay coefficients of variation were 10 and 11%, respectively.

Evaluation of mRNA encoding $P450_{scc}$

Experiment 1. To assess steady state levels of mRNA encoding $P450_{scc}$, corpora lutea were collected from normally cycling Western range ewes on days 3, 6, 9, 12 and 15 after observation of estrous behavior and on day 15 of pregnancy. RNA was isolated and analyzed for each luteal sample from individual ewes as described above.

Experiment 2. To determine whether the increase seen in $P450_{scc}$ mRNA from days 3 and 6 to day 9 in Experiment 1 was due to luteotropic hormonal stimulation, ewes were treated on day 10 post-estrous with 1000 IU hCG injected intravenously. hCG was used for this study because of its prolonged circulation in blood ($t_{1/2}$ = 23 h *vs* 25 min for oLH) and the repeated

demonstration that it binds to the LH receptor [24]. Blood was sampled at −2, −1, 0, 2, 4, 8, 12, 24 and 36 h, or until removal of the corpus luteum. Corpora lutea were collected 12, 24 or 36 h after hCG injection, immediately frozen on dry ice, weighed and stored at −70°C until RNA was extracted. Control ewes were injected with saline and corpora lutea collected at 12 or 36 h post-injection. Four animals were randomly assigned to each treatment or control group. However, due to lack of a corpus luteum in one hCG-treated ewe, the 12 h hCG treatment group consisted of only 3 animals. Luteal and serum progesterone concentrations were determined as described above.

Experiment 3. Since there is considerable variability in the stage of luteolysis of corpora lutea collected on day 15 of the ovine estrous cycle, levels of mRNA encoding $P450_{scc}$ were determined in an experimental model where luteolysis was precisely controlled with a known luteolytic regimen of $PGF_{2\alpha}$ [25]. Ewes were treated on day 10 post-estrus with two intramuscular injections of $PGF_{2\alpha}$ (lutalyse, 5 mg each) or saline at 4 h intervals. Corpora lutea were collected 1 or 8 h after the second prostaglandin injection or 8 h after the second saline injection ($n = 4$ per group) and analyzed as in Experiment 2. Blood samples were obtained hourly, starting 2 h prior to the first injection until removal of the corpora lutea.

In vitro experiments

Corpora lutea were surgically removed from ewes on day 10 of the estrous cycle, decapsulated, sliced and dissociated into single cell suspensions using 0.25% collagenase and 0.05% DNase [26]. Two enriched fractions of cells were obtained by centrifugal elutriation, containing primarily small (SLC, 10–20 μm) or large (LLC, 20–35 μm) steroidogenic cells [27]. Following quantitation using a hemocytometer under 40 × magnification, cells were washed three times with M199 and diluted to a concentration of 20–75 × 10^3/ml SLC or 6–22.5 × 10^3/ml LLC with M199 containing 20 mM HEPES, 4.2 mM $NaHCO_3$, 100 IU/ml penicillin G, 10 μg/ml streptomycin sulfate and 5% ram serum. Aliquots of 1 ml were placed into 24-well plates and allowed to attach for 18 h at 37°C in the presence of humidified room air. The medium was then removed and cells were washed three times with 1 ml serum-free M199. Fresh M199 was added along with exogenous substrate, lipoprotein, and hormone treatment to a total volume of 1 ml. Plates were gently agitated to mix the reagents, incubated at 37°C, and aliquots were removed at times indicated in the experiments. Progesterone was assayed in duplicate by radioimmunoassay [22] and expressed either as fg/cell/min or ng progesterone accumulated. The mean intra-assay coefficient of variation was 9%; inter-assay coefficient of variation was 14%.

Crude ovine lipoprotein was isolated from EDTA-anticoagulated sheep plasma by density gradient centrifugation; separation into high (oHDL) and low (oLDL) density fractions was achieved by agarose Bio-Gel A-5m chromatography [28]. Protein was quantitated using Biorad protein reagent and cholesterol by the Leibermann–Burchard procedure [29], both modified to assay 10 μl of sample. Purity of the oHDL fraction was evaluated by agarose electrophoretic analysis [30], which indicated less than 1% contamination of HDL lipid with LDL lipid.

Measurement of side-chain cleavage activity

Small or large ovine luteal cells, plated and washed as indicated, were used to measure $P450_{scc}$ activity. Linearity of enzyme activity over time was assessed using 50 × 10^3 or 15 × 10^3 small or large luteal cells, respectively, oHDL (100 μg cholesterol) and 22R-hydroxycholesterol substrate (30 μM). Progesterone production was evaluated at time points from 0–8 h after addition of reagents. The experiment was conducted on three occasions with 3 to 6 wells per replicate. Slopes representing progesterone produced at each time point were calculated using a Taylor series expansion [31] where x = elapsed time and $f(x)$ = accumulated progesterone; inclusion of preceding and following points yields a truer estimate of the dynamic process represented by progesterone production than does a simple slope calculation using two points. Unequal time intervals are also considered in the following equation:

$$\text{slope at } f_1 = (\alpha f_2 + (1 - \alpha) f_1 - f_0)/(h + \alpha j)$$

where h = time interval from f_0 to f_1, j = time interval from f_1 to f_2 and $\alpha = (h/j)^2$. The slopes thus calculated were compared by analysis of variance.

Linearity of the assay over a range of enzyme concentrations was assessed by plating 20–75 × 10^3 small, or 6–22.5 × 10^3 large luteal cells per well. Substrate 22R-hydroxycholesterol (30 μM) and oHDL (100 μg cholesterol/ml) were added, and concentration of progesterone in the medium evaluated at 0 and 2 h. The experiment was replicated on three occasions using two wells per point. Slopes calculated as described above were compared by analysis of variance.

Enzyme kinetics were examined using 50 × 10^3 small or 15 × 10^3 large luteal cells, 100 μg/ml oHDL cholesterol and 0 to 90 μM 22R-hydroxycholesterol substrate. Initial velocity (V_0) of the side-chain cleavage reaction was calculated as fg/cell/min at 2 h after addition of reagents. Values of $1/V_0$ were plotted against 1/substrate concentration. The experiment was replicated on four occasions using duplicate wells each time.

Effect of PKA and PKC upon $P450_{scc}$ activity

The effect of activation of the appropriate second messenger system upon side-chain cleavage activity was assessed by the following procedure. Small luteal cells (50 × 10^3/well) and large luteal cells

(15×10^3/well) were incubated with 100 μg/ml oHDL cholesterol and 30 μM 22R-hydroxycholesterol. LH, 200 ng/ml dissolved in M199, was utilized to activate the PKA second messenger system in small luteal cells. PMA, 100 nM dissolved in ethanol, was used to activate the PKC second messenger system of large luteal cells. The final ethanol concentration of 0.01% was added to control treatments in large luteal cells. To assess progesterone produced from the sum of endogenous substrate and HDL cholesterol, an additional series of wells containing 50×10^3 small luteal cells received no 22R-hydroxycholesterol but were exposed to HDL and 0 or 200 ng/ml LH. Another series contained 15×10^3 large luteal cells/well, no 22R-hydroxycholesterol, and HDL ± 100 nM PMA. Media were removed 0, 0.5, 1, 1.5, 2, 4, 6 and 8 h from triplicate wells and assayed for accumulated progesterone. Four replicates of the experiment were conducted. Data were analyzed by the general linear model of analysis of variance, in which treatment and time are considered together and separately.

RESULTS

$P450_{scc}$ messenger RNA

Northern blot analysis demonstrated specific binding of the $P450_{scc}$ cDNA probe to 25 μg of total RNA from luteal and adrenal tissue but not to RNA from liver, uterus, brain, kidney, heart or skeletal muscle. $P450_{scc}$ message existed primarily as a 2 kb species (Fig. 1).

Messenger RNA encoding $P450_{scc}$ was quantitated from Northern blots containing 20 μg of total RNA from individual corpora lutea. The amount of message

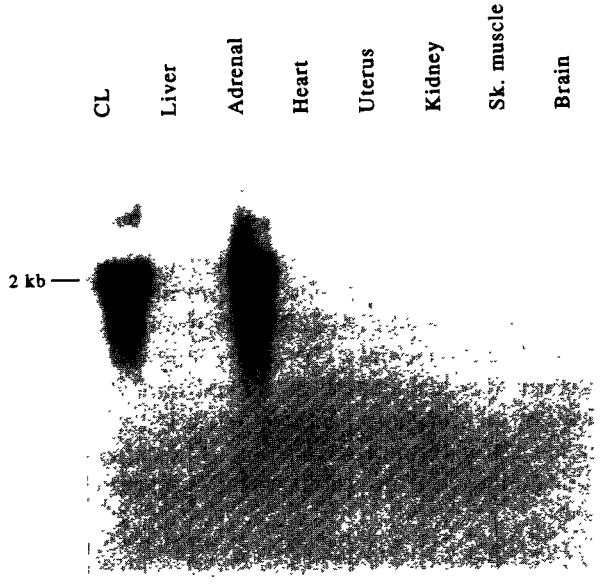

Fig. 1. Autoradiogram illustrating specific binding of cDNA encoding $P450_{scc}$ to 25 μg of total RNA isolated from steroidogenic and non-steroidogenic tissues. The minor band of radioactivity below the 2 kb band varied widely in intensity and was not included in quantitation of $P450_{scc}$ message.

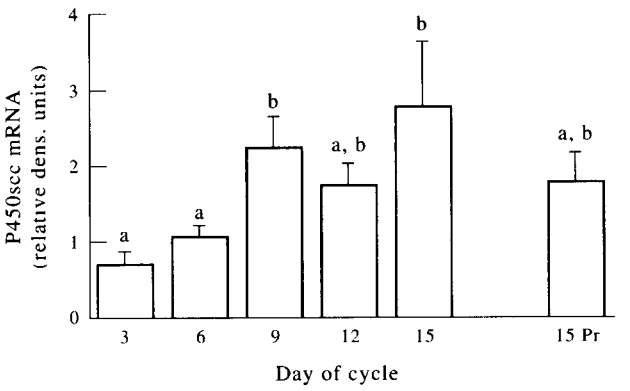

Fig. 2. $P450_{scc}$ mRNA measured in corpora lutea of cycling sheep. Data are expressed as mean ± standard error of the mean ($n = 4$ per group). Densitometric peak heights representing $P450_{scc}$ mRNA were divided by those of tubulin mRNA in the same samples to yield relative densitometric units. Significant differences ($P < 0.05$) are denoted by different letters (a,b).

encoding $P450_{scc}$ was similar on days 3 and 6 of the estrous cycle. On day 9 the mRNA concentration was higher (2-fold, $P < 0.05$) and remained elevated on day 15 of the cycle (Fig. 2). Mean luteal concentration of $P450_{scc}$ message on day 12 of the cycle was not different from that of any other day examined while concentrations on day 15 of pregnancy were similar to those found in luteal tissue from day 12.

No differences were observed in luteal weights between hCG- and saline-treated animals ($P < 0.05$). Concentrations of progesterone in luteal tissue at 36 h after treatment were not elevated over pretreatment levels but were elevated over those measured in the 36 h control group ($P < 0.05$, Fig. 3). Serum levels of progesterone were increased ($P < 0.05$) at 24 and 36 h post-treatment over pre-treatment levels, but were not different from the level measured in the 36 h control (Fig. 3). No significant differences in mRNA encoding

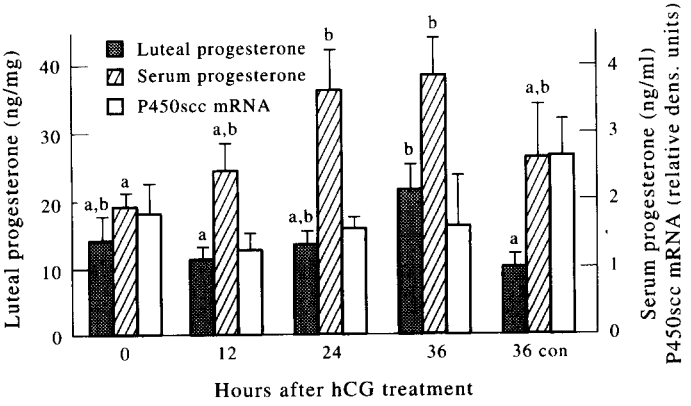

Fig. 3. Luteal tissue and serum concentrations of progesterone, and luteal mRNA encoding cytochrome $P450_{scc}$ following *in vivo* treatment with hCG ($n = 3$ or 4 per group). Quantity of mRNA encoding $P450_{scc}$ is expressed relative to that encoding tubulin. Significant differences ($P < 0.05$) are denoted by different letters (a,b).

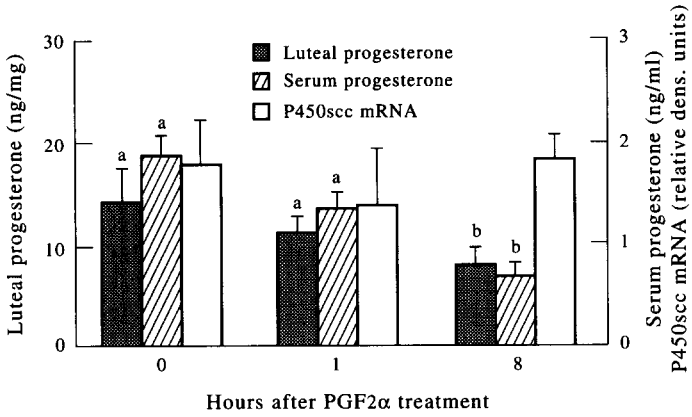

Fig. 4. Luteal tissue and serum concentrations of progesterone, and luteal mRNA encoding cytochrome $P450_{scc}$ following *in vivo* treatment with $PGF_{2\alpha}$ ($n = 4$ per group). The quantity of mRNA encoding $P450_{scc}$ is expressed relative to that encoding tubulin. Significant differences ($P < 0.05$) are represented by different letters (a,b).

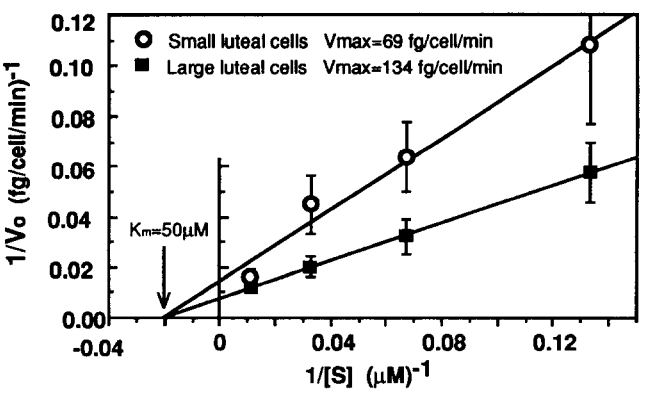

Fig. 5. Lineweaver–Burk plot representing kinetic parameters of $P450_{scc}$. V_{max} is calculated from the points at which the lines cross the Y-axis, K_m from the points of intersection with the X-axis. Mean progesterone and standard errors of the means were determined using measurements from four experiments.

$P450_{scc}$ were seen among any of the treatment groups ($P < 0.05$, Fig. 3).

Luteal weights did not differ among the control or $PGF_{2\alpha}$-treated groups at the times studied. However, at 8 h after the second injection both luteal progesterone and serum progesterone concentrations had decreased significantly ($P < 0.05$) when compared to both control and 1 h treatment levels (Fig. 4). Nevertheless, relative levels of mRNA encoding $P450_{scc}$ were not different among the three groups (Fig. 4).

Assay of side-chain cleavage activity

The assay of side-chain cleavage activity was linear over time for up to 8 h (data not shown). Comparison of the rates of progesterone synthesis at each time point by analysis of variance revealed no significant differences over the 8 h experiment, in either small or large plated luteal cells. Thus, the assay of side-chain cleavage activity, performed using 50×10^3 small luteal cells or 15×10^3 large luteal cells, 100 μg HDL cholesterol and 30 μM 22R-hydroxycholesterol per ml of culture medium is linear over a time span of 8 h.

Linearity of the side-chain cleavage assay over a range of cell numbers was demonstrated by comparison of the slopes calculated to represent progesterone production per cell number. Over a range of 20–75 $\times 10^3$ small luteal cells or 6–22.5 $\times 10^3$ large luteal cells, no significant differences were seen among the calculated rates of steroid synthesis (data not shown).

Kinetic data are presented in Fig. 5. A substrate concentration of 90 μM was the highest level utilized due to the limited solubility of the sterol. Data obtained at substrate concentrations $\geqslant 7.5$ μM were compared by the double-reciprocal Lineweaver–Burk plot (Fig. 5), from which K_m and V_{max} can be determined. The K_m determined in this manner was similar for both cell types (50 μM), while V_{max} of large cells exceeded that of small cells by nearly 2-fold (134 *vs* 69 fg/cell/min).

Effect of PKA and PKC upon $P450_{scc}$ enzyme activity

In both small and large luteal cells treated with HDL in the absence of exogenous substrate, activation of the PKA or PKC second messenger system, respectively, resulted in highly significant alterations ($P < 0.01$) in progesterone production (Fig. 6). In small luteal cell cultures, LH-stimulated progesterone production was 910% of that produced by unstimulated small luteal cells. PMA treatment of large luteal cells resulted in

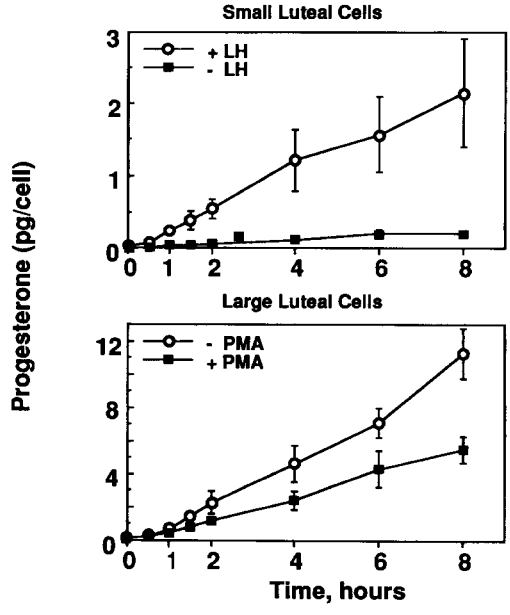

Fig. 6. Effect of LH (200 ng/ml) or PMA (100 nM) on production of progesterone by cultured small and large luteal cells in the presence of endogenous substrate and 100 μg HDL cholesterol. Mean progesterone accumulation and standard errors of the means represent data from four experiments. Differences between treated and control cultures were significant ($P < 0.01$) at times $\geqslant 90$ min.

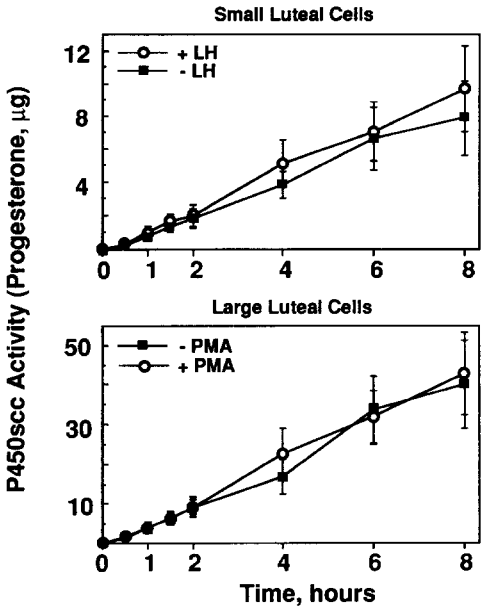

Fig. 7. Assay of cholesterol side-chain cleavage activity. In small luteal cells the PKA second messenger system was activated with LH; in large luteal cells the PKC second messenger system was activated by treatment with PMA. Cells were cultured with 100 μg HDL cholesterol and 30 μM 22R-hydroxycholesterol. Mean progesterone accumulation and standard errors of the means represent data from four experiments. No significant differences are apparent for treatment within time ($P < 0.05$).

51% reduction of steroid synthesis. The effects of second messenger activation upon activity of $P450_{scc}$ are shown in Fig. 7. The activity of cholesterol side-chain cleavage enzyme was determined from values of progesterone produced only from 22R-hydroxycholesterol. Progesterone produced from the sum of HDL and cellular stores of cholesterol (Fig. 6) was subtracted from each point. Neither treatment of small luteal cells with LH nor large luteal cells with PMA produced significant alteration ($P < 0.05$) in activity of the side-chain cleavage enzyme.

DISCUSSION

$P450_{scc}$ messenger RNA

Of ovine tissues examined, only corpus luteum and adrenal contained detectable quantities of mRNA encoding $P450_{scc}$. John *et al.* [17] previously noted the presence of $P450_{scc}$ mRNA in bovine adrenal cortex and corpus luteum, but not in heart, liver or kidney. The 2 kb size of mRNA transcript detected in this study was in agreement with that reported in bovine [17], human [10], rat [32] and monkey [33] luteal tissue.

Rodgers *et al.* [9] demonstrated detectable levels of mRNA encoding $P450_{scc}$ in corpora lutea classified by visual criteria as early, early-mid and late-mid tissue. Luteal tissue designated as regressing contained no detectable $P450_{scc}$ mRNA. In monkey, luteal mRNA encoding $P450_{scc}$ was maintained for the 15 day lifespan of the corpus luteum [33]. Similarly, Doody *et al.* [10] noted a high level of message encoding $P450_{scc}$ in human corpora lutea obtained late in the cycle. Data from the latter two studies indicated that only in regressing corpora lutea (obtained post menses) was the concentration of mRNA coding for $P450_{scc}$ reduced to undetectable levels. Although corpora lutea collected on day 15 of the cycle in the present study showed no gross signs of luteal regression, mRNA encoding 3βHSD was diminished by nearly 80% in this same luteal tissue [34] suggesting that the luteolytic process had been initiated.

Enhancement of cellular content of cytochrome $P450_{scc}$ in follicular cells by activation of PKA was demonstrated by Toaff *et al.* [2], Funkenstein *et al.* [3], Trzeciak *et al.* [35], Voutilainen *et al.* [36], Golos *et al.* [5], Mcallister *et al.* [37] and Urban *et al.* [6]. Similar increases are seen upon luteinization in response to the LH surge [4, 38]. Maintenance of progesterone secretion by luteinized rat pre-ovulatory follicles in culture without LH stimulation [39] suggest that $P450_{scc}$ is quite stable following luteinization.

Treatment of sheep with hCG on day 10 of the estrous cycle failed to alter the quantity of mRNA coding for $P450_{scc}$ after 36 h. Although serum concentrations of progesterone were approximately doubled at 24 and 36 h post-hCG treatment ($P < 0.05$ compared to 0 h controls), these levels only tended ($P < 0.05$) to be different from those observed in the 36 h control group. This finding is similar to those of Suter *et al.* [40] who increased serum levels of LH over 200-fold but detected less than a 50% increase in serum levels of progesterone between 10 min and 2 h after injection of LH. Since large luteal cells secrete over 85% of total luteal progestin [41] but do not respond to LH, this observation is not surprising. There are no receptors for FSH detectable in the ovine corpus luteum under conditions where they are easily quantified in bovine corpora lutea [42]. Thus, FSH cannot be considered a candidate for regulation of luteal $P450_{scc}$ activity in ovine luteal cells. It is thus concluded that tropic hormone stimulation did not increase luteal content of mRNA encoding cytochrome $P450_{scc}$.

In the current study, concentrations of both serum and luteal progesterone were significantly depressed 8 h after the second injection of $PGF_{2\alpha}$. In spite of this indication of PKC inhibition of steroidogenesis, luteal levels of mRNA encoding cytochrome $P450_{scc}$ were not decreased. Therefore, acute regulation of progesterone synthesis through either activation of PKA by hCG or activation of PKC by $PGF_{2\alpha}$ does not appear to be due to alterations in the level of mRNA encoding cytochrome $P450_{scc}$.

Effect of PKA and PKC upon $P450_{scc}$ enzyme activity

In the present study, conversion of 22R-hydroxycholesterol to progesterone reflects activity of $P450_{scc}$, and activation of PKA, which increased progesterone

production 9-fold, did not affect the activity of side-chain cleavage enzyme. Enhanced side-chain cleavage enzyme activity after PKA activation has generally reflected increased enzyme mass following extended stimulation [2, 43, 44]. In most cases where PKA activation enhances side-chain cleavage enzyme activity, the time span studied is far longer than that required to observe effects of LH on progesterone production. By 30 min (Fig. 6) progesterone produced from endogenous substrate in LH-stimulated small luteal cells was 7.6-fold of that measured in unstimulated cells. Thus, while enhanced enzyme activity (itself a reflection of increased enzyme concentration) appears to result from long-term stimulation of progestin synthesis, such enhancement is not involved in acute stimulation.

PKC activation has also been shown to exert an effect on side-chain cleavage enzyme activity. Wiltbank *et al.* [13], using ovine large luteal cells, noted a 28% decrease in progesterone production by cells treated with 25-hydroxycholesterol and PMA, compared to a 50% attenuation from cells utilizing substrate from endogenous sources. Veldhuis and Demers [12] reported 80% lower conversion of exogenous hydroxylated substrates in porcine granulosa cells treated with PMA. In neither of these studies, however, were cells treated with lipoprotein. Without enhanced entry of substrate, decreased progesterone production from 22R-hydroxycholesterol following PMA treatment may simply reflect a limitation of endogenous substrate.

Grusenmeyer and Pate [14] assessed side-chain cleavage activity in bovine luteal cells in the absence of lipoprotein, but detected no difference in conversion of 25-hydroxycholesterol to progesterone following 7 days of $PGF_{2\alpha}$ treatment at 10 ng/ml (28 nM). In the current study, 1 h of PMA treatment produced a decrease in progesterone production of 30%, while at 90 min the loss in progesterone production was 42% (Fig. 6); the latter difference was significant at $P < 0.01$. Thus a loss in side-chain cleavage enzyme activity should be apparent by 90 min of PMA treatment, if such alteration is the mechanism of decrease in progesterone production. Extended pretreatment by activators of PKC might actually deplete PKC with resultant absence of effects of additional $PGF_{2\alpha}$ or PMA. In fact, Wiltbank *et al.* [13] showed 82 and 93% decrease in PKC activity in small and large ovine luteal cells, respectively, following treatment for 12–18 h with 1 nM PMA.

On the basis of the data obtained, it is concluded that while stimulation of progesterone production follows activation of the PKA second messenger system, and inhibition results from activation of the PKC system, these actions are not effects of changes in cytochrome $P450_{scc}$ enzyme activity. It is possible, then, that steroidogenesis is altered through effects of PKA and PKC activation upon availability of substrate, transport of substrate to the mitochondria or transport of substrate within mitochondria to cytochrome $P450_{scc}$.

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