Dependence on Prolactin of the Luteolytic Effect of Prostaglandin $F_{2\alpha}$ in Rat Luteal Cell Cultures[1]

Anna Zetser,[3] Tatiana Kisliouk,[3] Emma Ivakin, and Michal Lahav[2]

Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa 31096, Israel

ABSTRACT

Luteal regression is a multistep, prolonged process, and long-term luteal cultures are required for studying it in vitro. Cell suspensions from ovaries of superovulated rats were enriched with steroidogenic cells, seeded on laminin or fibronectin, and maintained in defined medium for up to 10 days. Progesterone secretion was much lower than that of 20α-dihydroprogesterone, a product of 20α-hydroxysteroid dehydrogenase (20α-HSD). Prolactin added throughout the incubation period gradually increased the percent progesterone out of total progestins to fourfold, while reducing 20α-HSD mRNA by 73%. Luteinizing hormone accelerated the establishment of higher percent progesterone by prolactin but by itself had no effect. Prolactin did not increase total progestin production or cytochrome P450 side-chain cleavage ($P450_{scc}$) mRNA. Cell viability was unaffected by prolactin and/or LH. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was added 7–8 days after seeding. In prolactin-treated cells, $PGF_{2\alpha}$ reduced steroidogenesis after 4–45 h, and at 45 h total progestins and $P450_{scc}$ mRNA were reduced by 45%. At 8–45 h $PGF_{2\alpha}$ reduced the percent progesterone out of total progestins, and at 45 h 20α-HSD mRNA was doubled. In contrast, in prolactin-deprived cultures, $PGF_{2\alpha}$ had little effect on total progestins or 20α-HSD mRNA but doubled $P450_{scc}$ mRNA. Phospholipase C activity was stimulated by $PGF_{2\alpha}$ regardless of prolactin. Thus, when prolactin-treated, our cultures are a good model for mature corpora lutea challenged with $PGF_{2\alpha}$; the finding that without prolactin $PGF_{2\alpha}$ has an alternative set of actions could help in identifying the signaling pathways of $PGF_{2\alpha}$ responsible for its luteolytic effects.

corpus luteum, luteinizing hormone, progesterone, prolactin, steroid hormones

INTRODUCTION

In the multistage process of luteolysis, a decrease in progesterone production marks the early phase, whereas structural involution occurs later [1–3]. The effect of pregnancy to prolong luteal function is especially dramatic in the rat: two daily surges of pituitary prolactin (PRL), induced by copulation, and subsequently placental lactogens, extend luteal functional life span from 2 to 20 days and augment plasma progesterone. Endogenous PRL also underlies the prolonged luteal function in the various models of pseudopregnancy [3–5]. Paradoxically, corpora lutea (CL) that had undergone functional luteolysis actually require PRL to proceed through structural regression [5, 6]. Luteinizing hormone, the central luteotropin in many species, is essential for luteinization in the rat as well but later becomes dispensable. Moreover, LH applied to ovaries of pregnant rats in situ failed to acutely increase progesterone production [3, 4, 7, 8]. In contrast, in rat luteal cell suspensions, LH was very effective in rapidly stimulating progesterone synthesis [9–11].

In the pregnant or pseudopregnant rat, administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was shown to initiate luteolysis. Prostaglandin $F_{2\alpha}$ given to pseudopregnant rats reduced, after 15 min, the plasma levels of progesterone and of its inactive metabolite, 20α-dihydroprogesterone (20α-DHP) [12, 13]. With a similar time course, $PGF_{2\alpha}$ inhibited in vitro the LH-stimulated progesterone and/or cAMP production in isolated intact rat CL or luteal cell suspensions [9, 14]. Eight to twelve hours after $PGF_{2\alpha}$ injection, increased activity of 20α-hydroxysteroid dehydrogenase (20α-HSD), the luteal enzyme that converts progesterone to 20α-DHP, was observed [12, 13, 15]. In functional rat CL this enzyme is maintained at a very low level by PRL [4, 15, 16]. Several years ago, 20α-HSD cDNA was cloned, gene organization and promoter sequence were determined, and inhibition by PRL of 20α-HSD expression was demonstrated in vivo and in vitro [16–18]. In contrast, stimulation by $PGF_{2\alpha}$ of 20α-HSD expression has not been demonstrated in luteal tissue in vitro.

Interestingly, mature CL were shown in several species to be much more sensitive to administered $PGF_{2\alpha}$ than young CL [19]. In pseudopregnant rats this phenomenon was demonstrated both in vivo [15] and in vitro [20, 21].

Long-term luteal cell cultures would be most useful for studying the regulation of the entire process of luteolysis, including structural regression. However, rat luteal tissue proved difficult to culture for long periods; thus, primarily short-term hormonal effects have been studied in vitro, typically by incubating luteal cell suspensions or isolated CL for up to a few hours. Cultured cells were usually maintained for 1–3 days only [22]. Many years ago we cultured a whole dispersate of rat CL of pregnancy in serum-replete medium for up to 12 days. We found that PRL added in vitro partly suppressed the spontaneous rise in 20α-HSD activity that occurred in vitro [23]. More recently, models of rat luteal cell cultures were developed and characterized in several laboratories; these include luteinized granulosa cells maintained with 1% serum [18, 24], immortalized large luteal cells [25], and two types of serum-free, long-term (10 days or more) cultures of luteal steroidogenic cells [7, 26]. Nelson et al. [26] prepared cell suspensions from Day 3 CL of pregnancy by a particularly gentle procedure and fractionated them before seeding into large steroidogenic, small steroidogenic, and nonsteroidogenic cells by elutriation. Aten et al. [7] cultured, on laminin or fibronectin, luteinizing granulosa cells, which in the presence of

LH, PRL, and insulin-like growth factor-1 underwent advanced luteinization in vitro. The serum-free cultures described here are largely based on the latter two studies but were prepared from 4-day-old luteal tissue of superovulated pseudopregnant rats. The purpose of this study was to investigate in vitro those effects of PGF$_{2\alpha}$ that in vivo take at least several hours to become apparent.

MATERIALS AND METHODS

Materials

Equine CG (Synchroject) was a product of Vetimex (Bladel, Holland). Human CG (CR-119, 11 600 IU/mg), bovine LH (NIAMDD-bLH-4 and USDA-bLH-B-5), and ovine prolactin (NIADDK-o-PRL-16) were a generous gift from the National Institutes of Health (NIH), Bethesda, MD. Dulbecco modified Eagle medium (DMEM; low glucose, without sodium bicarbonate, 10×), DMEM:F12 (1:1, with 15 mM Hepes and 1.2 g/L sodium bicarbonate), inositol-free DMEM:F12, antibiotics mixture 100× (containing penicillin, streptomycin, and amphotericin B), gentamycin, nystatin, bovine fibronectin, trypsin (0.25%)-EDTA (0.05%) solution, and donor horse serum were purchased from Biological Industries, Kibbutz Beit-HaEmek, Israel. Collagenase A, dispase grade II, DNAse I, Apoptotic DNA Ladder Kit (cat. no. 1835246), Random Primed DNA Labeling Kit (cat. no. 1004760), and laminin (no longer produced) were products of Boehringer Mannheim (later Roche Diagnostics GmbH), Mannheim, Germany. The following were purchased from Sigma (St. Louis, MO): bovine insulin (I-6634), bovine apotransferrin (T-1428), sodium selenite, BSA (A-2153), salmon sperm DNA, calf thymus DNA, bisbenzimide (Hoechst H33258), myoinositol, progesterone, 20α-DHP, 123-base pair (bp) DNA ladder. Rabbit antiprogesterone 11α-BSA serum and rabbit antiserum to 20α-dihydroprogesterone-3-thyroglobulin, products of BioMakor (Rehovot, Israel), were obtained from Sigma. Agarose and Trizol reagent were products of Gibco-BRL. Cyclic AMP RIA kit (cat. no. 1117) was manufactured by Immunotech (Marseille, France). Dowex (AG 1×8 200/400 mesh) was a product of Bio-Rad (Hercules, CA). [1,2,6,7-^{3}H]Progesterone and [^{32}P]dCTP were purchased from Amersham Pharmacia Biotech UK Ltd. (Amersham Place, England), whereas [1,2-^{3}H]-20α-DHP and myo-[2-^{3}H]inositol were products of DuPont (New England Nuclear Corporation, Boston, MA). Scintillation solutions were CytoScint (ICN Biomedicals, Costa Mesa, CA) for steroids, and OptiPhase HiSafe (Wallac Oy, Turku, Finland) for inositol and its derivatives. Rat cytochrome P450 cholesterol side-chain cleavage enzyme (P450$_{scc}$) DNA probe (1.2 kilobases [kb]) in pGEM 3Z was kindly donated by Dr. JoAnne S. Richards (Baylor College of Medicine, Houston, TX) [24]. 20α-Hydroxysteroid dehydrogenase cDNA (1.2 kb) in pBluescript SK was a generous gift of Dr. Geula Gibori, The University of Illinois at Chicago, Chicago, IL [16].

Animals and Cell Cultures

Sprague-Dawley female rats (Harlan Labs, Jerusalem, Israel) were maintained under controlled temperature and light (12L:12D), with free access to rat chow and water. All experiments were approved by the Committee for Supervision of Animal Experiments of the Technion, Israel Institute of Technology. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the NIH. Rats (29 ± 1 days old) were given 60 IU (in earlier experiments 50 IU) eCG, followed by 25 IU hCG 3 days later (both administered s.c. in 0.2 ml saline). After an additional 4 days, rats were killed by decapitation. Four-day-old luteinized ovaries were superior to 7- and 2-day-old tissue (steroidogenesis deteriorated rapidly in cultures prepared from the former, and cell yield was low after dispersing the latter).

Each pair of ovaries was removed into 10 ml DMEM (plus nonessential amino acids and 25 mM Hepes), trimmed of foreign tissue, rinsed twice in the same medium, and weighed (in the second-rinse preweighed vial). The dissociation procedure was essentially that of Nelson et al. [26]. All solutions used, except for the Percoll gradients, were prewarmed to 37°C. Finely cut tissue (8–10 pieces/ovary) was collected in 50-ml sterile conical tubes containing dissociation solution (DS), comprised of calcium- and magnesium-free Hanks buffered salt solution, 25 mM Hepes, 3 mM sodium bicarbonate, 1 g/liter glucose, and 2% BSA, pH 7.4. Freshly prepared enzyme mixture (in DS) was then added to all tubes, to give the following final concentrations (µg/ml) in the digestion mixture: collagenase, 0.17; dispase, 0.5; DNase, 0.15; tissue concentration was 100 mg/ml (up to 10 ml/tube). The capped test tubes (with a 100% oxygen atmosphere inside) were incubated for 30 min at 37°C in a shaking water

bath, centrifuged (160 × g, 5 min), and the cells resuspended in digestion mixture. Four such digestion cycles were applied. To secure gentle cell handling, 40-cm pieces of glass tubing (inner diameter 3.5 or 2.5 mm, equipped with a silicone bulb) were used throughout instead of pipettes. The dispersate was then exposed (10 min at room temperature) to EDTA (0.02% in DMEM salts, minus calcium, plus glucose, Hepes, and BSA, pH 7.4), and rinsed three times in DS. Cells were filtered through an 80-µm nylon mesh and fractionated on a continuous Percoll gradient (500 × g, 23 min; cells from three to four ovaries per tube). The gradient was prepared by precentrifugation of 40% Percoll in 0.9% saline (8 ml per 12-ml polycarbonate tube) for 60 min at 20 000 × g. The thick band of steroidogenic cells at a density of approximately 1.03 was collected into test tubes containing DS, rinsed twice with DS and once with culture medium, resuspended in culture medium, and counted. Under the microscope, most cells looked steroidogenic, and debris was virtually absent. The culture medium was DMEM:F12 (1:1), containing 15 mM Hepes and 1.2 g/liter sodium bicarbonate, and supplemented with 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 8 ng/ml sodium selenite, human high-density lipoprotein (HDL: 25 µg protein/ml, prepared in the laboratory of Dr. Michael Aviram, Department of Biochemistry [27]), 0.125% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, 50 µg/ml gentamycin sulfate, and 12.5 U/ml nystatin. Seeding density was around 100 000 cells/cm^2. For experiments aimed at progestin and DNA determination, cells were seeded in 24-well plates (area 2 cm^2/well, 1 ml medium/well), with laminin or fibronectin (2 and 3 µg/cm^2, respectively). For the determination of phosphoinositide metabolism and DNA fragmentation, cells were seeded in six-well plates (3 ml medium/well, fibronectin 6 µg/cm^2). For mRNA measurements, cells were seeded in 60-mm dishes (10 ml medium; fibronectin, 6 µg/cm^2). Prolactin (200 ng/ml) and/or LH (100 pg/ml [7]) were present in the incubation medium of some cultures throughout the incubation period (up to 10 days).

Medium was removed 2 days after seeding and discarded. Medium was then changed every 2 days (occasionally after 1 day, never after 3). Spent medium was saved for progestin analysis as indicated, and stored at −20°C. At the end of incubation, the emptied wells (of 24-well plates) were sometimes rinsed twice with cold saline and the cell-containing plates stored at −20°C until analyzed for DNA.

Progestin and DNA Analysis

Progesterone and 20α-DHP were determined by RIAs already described, using tritium-labeled steroids [28]. For assaying progestins in serum, samples were extracted in ether first. Assays were run in duplicate (standard curves in triplicate).

DNA was determined fluorometrically [29]; samples were prepared by scraping cells from each well into 1.5 ml of assay buffer, followed by sonication.

DNA Fragmentation Assay

Cells subjected to each treatment (five to six wells) were collected by trypsinization (1 ml trypsin for 6 min at 37°C, then 0.25 ml serum), centrifuged (160 × g, 5 min), and resuspended in 200 µl PBS. DNA was isolated and electrophoresed on ethidium bromide-containing 2% agarose gels according to instructions of the manufacturer of the Apoptotic DNA Ladder Kit.

Estimation of Phosphoinositide Metabolism

The procedure used was based on published studies [30, 31]. After 8 days in culture with or without PRL, cells were rinsed twice with inositol-free DMEM:F12 medium, incubated for 20 h in 2 ml inositol-free culture medium with [^{3}H]inositol (5 µCi/ml), and rinsed three times with culture medium containing 10 mM inositol (the last rinse's medium removed after 30 min). Culture medium supplemented with 10 mM LiCl was then added, and 15 min later PGF$_{2\alpha}$ was added where indicated (to give 100 nM). Fifteen minutes later the medium was discarded, the wells were rinsed twice with ice-cold saline, and cold 10% trichloroacetic acid (TCA) was added (1.5 ml/well). After at least 30 min at 4°C, cells were scraped with a silicone rubber policeman, and the contents of four similarly treated wells were combined and centrifuged at 4°C (1900 × g, 15 min). The supernatants were treated as described, including fractionation on 1-ml Dowex (AG 1×8 200–400 mesh) columns [30], except that inositol bisphosphate elution solution contained 600 mM ammonium formate [31]. With each elution solution, three washes (6, 3, and 3 ml) were applied. Three-milliliter eluates were counted in 9 ml of OptiPhase. When the TCA-insoluble fractions (which contain the phospholipids) were dissolved

FIG. 1. Rat luteal cell culture. Cells were seeded on fibronectin and incubated in the presence of PRL for 6 days. The live culture was photographed using an inverted microscope with total magnification (ocular plus objective) of ×160.
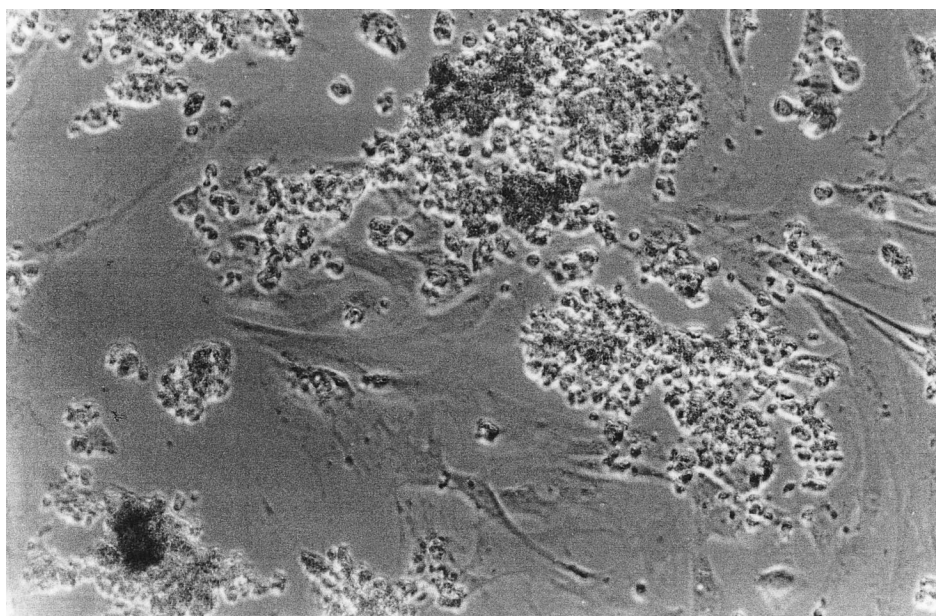
in NaOH and counted, it was found that PRL reduced [³H]inositol incorporation into phosphoinositides (PGF$_{2\alpha}$ had no effect). Interestingly, total radioactivity in the TCA-soluble fractions, in which free inositol dominated, correlated closely with total TCA-insoluble radioactivity. Thus, for each sample, the radioactivity in each soluble fraction was normalized to the total acid-soluble radioactivity.

Northern Blots

Cells subjected to each of four treatments (five to eight dishes/treatment) were collected by trypsinization and centrifugation (160 × g, 5 min), transferred to a 1.5-ml microfuge tube in 1 ml 0.9% saline, and after removal of 2–5 μl for counting, the cells were centrifuged. Trizol reagent was added to the sediment (1 ml per 7 million cells), and total RNA was isolated according to the manufacturer's instructions, with one additional rinse after RNA precipitation with isopropanol. Specifically, 80 μl water, 220 μl ethanol, and 30 μl sodium acetate (3 M, pH 5.2) were sequentially added to the sediment, and after overnight storage at −20°C the tubes were centrifuged (12 000 × g, 15 min), the sediment rinsed with 400 μl of 75% ethanol, and then dissolved in 14 μl water, 2 μl of which were used for RNA quantification. The water used throughout had been autoclaved with diethyl pyrocarbonate (10 μl/100 ml). Of each RNA sample, 20 μg were subjected to electrophoresis on a formaldehyde-containing 1.2% agarose gel. Electrophoresis, transfer to a nylon membrane (MSI, Westboro, MA), and hybridization with ³²P-labeled probes were conducted as described [32]. Complementary DNA probes of 20α-HSD and P450$_{scc}$ were labeled with [³²P]dCTP, using the Random Primed DNA Labeling Kit according to the manufacturer's instructions. Band radioactivity was estimated using a phosphorimager. These values were normalized to the optical density of the 18S rRNA band in the gel photograph, quantified by a densitometer.

TABLE 1. Effect of continuous hormonal treatment on the amount of DNA per well at the end of incubation.

Hormone	Normalized DNA contents[a]	
	Fibronectin	Laminin
Control	1.00 (4)	1.00 (4)
LH (100 pg/ml)	1.06 ± 0.13 (4)	0.81 ± 0.06 (4)
PRL (200 ng/ml)	1.05 ± 0.10 (4)	0.98 ± 0.03 (4)
LH + PRL	1.01 ± 0.01 (3)	0.97 ± 0.04 (2)

[a] Within each experiment, and for either matrix, averages were obtained for each hormonal treatment and normalized to that of the control. Means ± SEM (or range) were then obtained for two to four experiments (numbers in parentheses). Mean absolute DNA values in the controls seeded on fibronectin and laminin were 0.75 ± 0.26 and 1.42 ± 0.55, respectively.

Statistics

SPSS for Windows, releases 6.0 and 9.0 (SPSS Inc., Chicago, IL) was used for statistical calculation. One-way ANOVA with Student-Newman-Keuls test, t-test for independent samples, and paired t-test were used as appropriate; transformation of data was performed when the Levene test for homogeneity of variance indicated it. Rarely (Figs. 2, A and C, Days 3–4), nonparametric tests (Kruskal-Wallis and Mann-Whitney) had to be used. For comparisons among the three incubation periods in Figure 2, Friedman and Wilcoxon signed-rank test was used. Unless stated otherwise, means ± SEM are presented, and $P \leq 0.05$ is considered significant. In complex experiments, in which each group of wells participated in two or three comparisons, statistical significance for t-test was granted with $P \leq 0.03$ and $P \leq 0.02$, respectively.

RESULTS

Culture Morphology

Although completely dispersed at the time of seeding, the cells tended to reaggregate so that 12 h after seeding aggregation as well as attachment were well advanced. On the first medium change (2 days after seeding) all floating cells and aggregates were removed, and detachment was negligible during the following week. Some of the aggregates were multilayered, with the thickest spots looking the darkest (Fig. 1). Cultures with all hormonal treatments looked indistinguishable.

Effect of Incubation Conditions on the Amount of DNA per Well

Table 1 shows that the amount of DNA per well 6–8 days after seeding was not affected by the continuous presence of LH and/or PRL. The extracellular matrix, however, did make a difference. In the three experiments within which fibronectin and laminin were compared, the amount of DNA in wells supplemented with laminin was larger by 30% ($P < 0.02$), 108% ($P < 0.001$), and 53% ($P < 0.001$); the absolute values on fibronectin, which served as 100%, were 0.55, 1.03, and 1.50 μg DNA/well, respectively.

Effect of Continuous Treatment with LH and PRL on Progestin Production

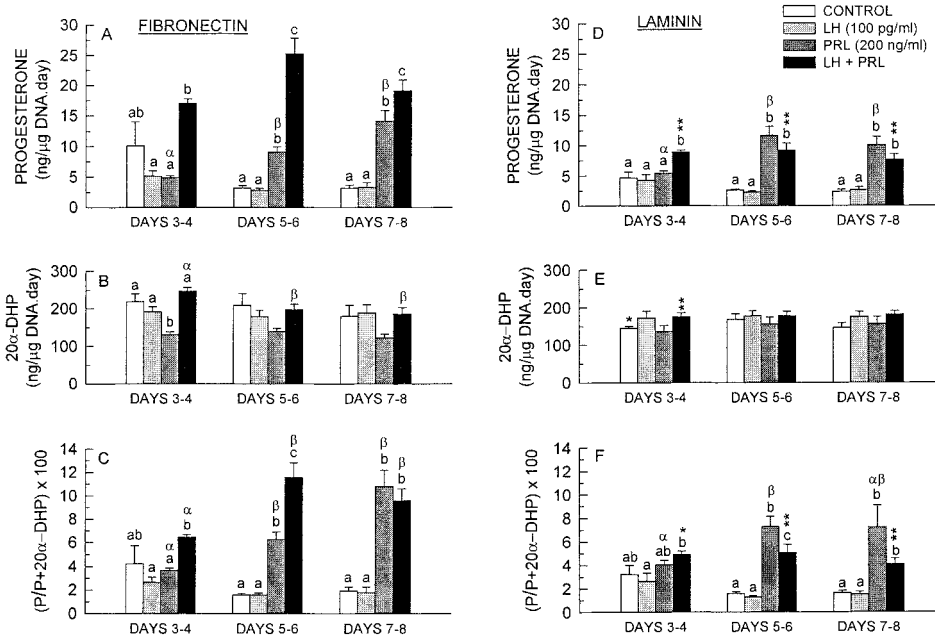Figure 2 describes an experiment, in which LH and/or PRL were added at the time of seeding to cells seeded on

FIG. 2. Time course of progestin production by cells seeded on fibronectin (**A–C**) or laminin (**D–F**) and incubated for 8 days with LH and/or PRL as specified. Medium was changed every 2 days, and progesterone and 20α-DHP were determined in the spent medium. For each hormonal treatment, within each matrix, means ± SEM were calculated (n = 8 samples, each comprised of combined medium from two wells). Progesterone (**A**, **D**) and 20α-DHP (**B**, **E**) are presented as ng/μg DNA.day. The DNA used for normalization is the mean DNA content measured at the end of the incubation in the wells of the respective treatment. The percent progesterone out of total progestins is also presented (**C**, **F**). Latin letters: Comparison among hormonal treatments at the same incubation period, on the same matrix. Greek letters: Comparison among incubation periods within the same hormonal treatment, on the same matrix. *$P < 0.005$, **$P < 0.001$, compared to cells seeded on fibronectin and incubated with the same hormonal treatment, for the same incubation period.

either fibronectin (Fig. 2, A–C) or laminin (Fig. 2, D–F). Progesterone (Fig. 2, A and D) and 20α-DHP (Fig. 2, B and E) in the incubation medium were monitored throughout Days 3–8 of culture. After compensating for the higher attachment and/or survival of cells on laminin by expressing the progestins as ng/day per μg DNA, progestin production on the two extracellular matrices was mostly (although not entirely) similar. In the control wells, progesterone secretion tended initially to decrease but then stabilized. Addition of LH alone had no effect whatsoever, presumably because the expression of LH receptors requires PRL [33]. Prolactin brought about a large, but slowly developing, increase in progesterone. In contrast, PRL in combination with LH stimulated progesterone production already on Days 3–4, but later there was no further increase. Consequently, the advantage of treatment with LH in addition to PRL gradually diminished (in cells seeded on fibronectin; on laminin, LH even became disadvantageous; Fig. 2, A and D). Thus, beyond the early series of experiments, LH was no longer used for long-term exposures.

20α-Dihydroprogesterone was always produced in larger amounts than progesterone in all our luteal cultures. 20α-Dihydroprogesterone did not vary significantly with treatment, the only exception being a tendency of PRL alone to suppress 20α-DHP, and hence total progestin production, in cells seeded on fibronectin (Fig. 2, B and E). Thus, the percent progesterone out of total progestins (Fig. 2, C and F) followed a pattern very similar to progesterone. The increase by PRL of the percent progesterone suggests suppression of 20α-HSD activity.

A striking feature of these cultures was the great stability of total progestin production with time: virtually no decrease was observed beyond Day 2 of incubation.

Each of the findings of Figure 2 just described was confirmed in at least two more experiments. The effects of PRL in cultures seeded on fibronectin and maintained for 6–10 days were summarized from 15 independent experiments: PRL inhibited total progestin production by 22 ± 8% ($P < 0.02$) and increased the percent progesterone out of total progestins to 394 ± 81% of the control ($P < 0.005$).

Effect of PGF$_{2\alpha}$ on Progestin Production

Exposure to PGF$_{2\alpha}$ was regularly initiated 7–8 days after seeding, with the purpose of allowing the cells to reach the new steady states shaped by treatment with PRL and/or LH, or deprivation thereof. The cultures described in Figure 2 (except for those maintained on LH plus PRL) were exposed on Day 8 to PGF$_{2\alpha}$ (10 μM) and/or a high concentration of LH (100 ng/ml) for 8 h. Because progestin patterns were very similar in cells seeded on fibronectin and laminin, data from all wells subjected to the same hormonal treatment were combined, regardless of the extracellular matrix. Progesterone production during this 8-h incubation is shown in Figure 3.

In PRL-deprived cells, exposure to the high concentration of LH had no effect, but in PRL-treated cells proges-
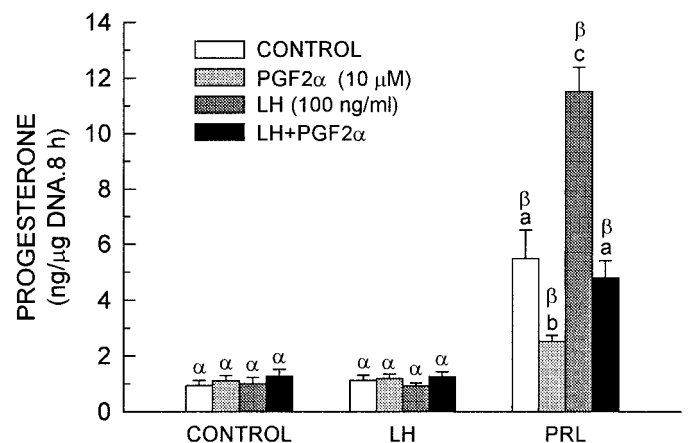

FIG. 3. Progesterone production during an 8-h exposure to PGF$_{2\alpha}$ and/or a high concentration of LH (100 ng/ml) at the end of an 8-day incubation with a low concentration of LH (100 pg/ml), or PRL (200 ng/ml), or no hormones. The cultures are the same as those presented in Figure 2. Wells subjected to the same hormonal treatment were combined, regardless of matrix. Means ± SEM (n = 8 wells). Latin letters: Comparison among acute hormonal exposures, in cells subjected to the same long-term hormonal treatment. Greek letters: Comparison among long-term hormonal treatments, in cells acutely exposed to the same hormone.
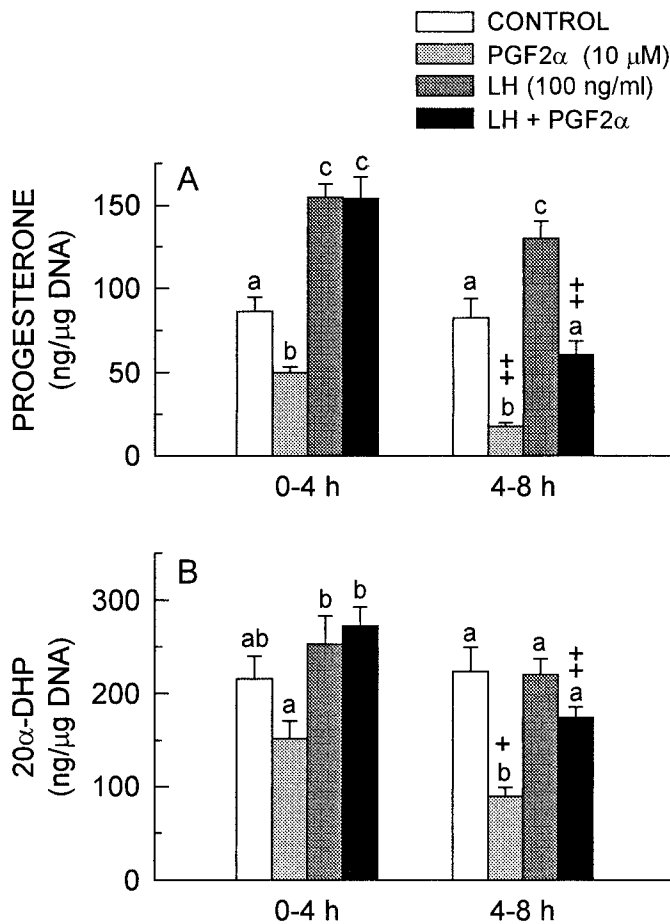
FIG. 4. Production of progesterone (**A**) and 20α-DHP (**B**) by cells seeded on laminin, maintained for 7 days with PRL (200 ng/ml), and then exposed to $PGF_{2\alpha}$ and/or a high LH concentration. Medium was changed after 4 h, the incubation continued with identical treatment for another 4-h period, and steroidogenesis was evaluated separately for each of the two incubation periods. Means ± SEM (n = 6 wells). Letters: Comparison among treatments within the same incubation period. $+P < 0.005$, $++P < 0.001$ compared to the same well during the first 4-h incubation period.

FIG. 5. Production of progesterone (**A**) and 20α-DHP (**B**) in cells exposed to various concentrations of $PGF_{2\alpha}$. The experiment and experimental design were the same as in Figure 4. Letters: Comparison among the various concentrations of $PGF_{2\alpha}$, within the same incubation period. $+P < 0.005$, $++P < 0.001$ compared to the same well during the first 4-h incubation period.

terone secretion was doubled. Prostaglandin $F_{2\alpha}$ applied to PRL-treated cells markedly inhibited progesterone synthesis (both basal and LH-stimulated). However, quite surprisingly, $PGF_{2\alpha}$ was not inhibitory in the absence of PRL (Fig. 3). These results were verified in two additional cell preparations. In one of these experiments, 7-day-old cultures seeded on laminin were exposed to $PGF_{2\alpha}$ for a total of 8 h, but medium was changed after 4 h, so that the effects of $PGF_{2\alpha}$ after shorter (4 h) and longer (8 h) exposures could be compared. Figure 4 shows the results in PRL-treated cells. During the second incubation period (4–8 h), $PGF_{2\alpha}$ inhibited both basal and LH-stimulated progesterone synthesis, as observed in the previous experiment. However, during the first 4 h, LH protected progesterone from the inhibitory effect of $PGF_{2\alpha}$ (Fig. 4A). 20α-Dihydroprogesterone followed a pattern similar to that of progesterone, but the responses were more blunted (Fig. 4B); this was true also for the experiment of Figure 3 (not shown).

Within the same experiment, the effect of lower concentrations of $PGF_{2\alpha}$ (0.01–1 μM) on basal steroidogenesis was also tested (Fig. 5). Regarding both progesterone and 20α-DHP, all doses of $PGF_{2\alpha}$ were similarly effective, with greater inhibition at the second incubation period. 20α-Dihydroprogesterone was inhibited to a lesser extent than pro-

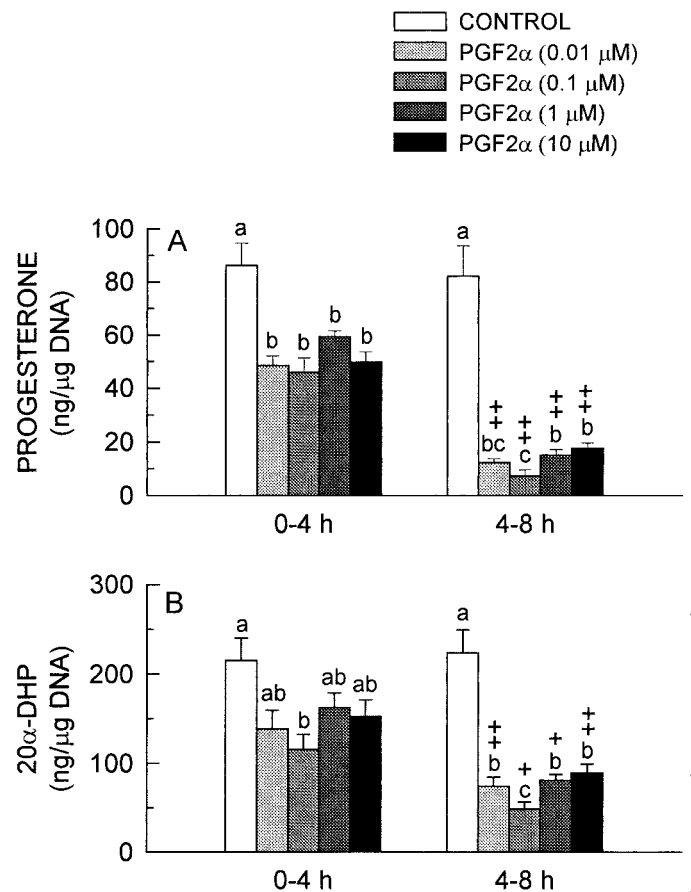gesterone. However, during the first 4 h the difference was small, and thus the percent progesterone out of total progestins was not significantly reduced by $PGF_{2\alpha}$: the values for 0, 0.01, 0.1, 1, and 10 μM $PGF_{2\alpha}$ were 30.8 ± 1.6, 27.1 ± 2.1, 29.0 ± 2.0, 27.4 ± 1.9, and 25.6 ± 2.3%, respectively. In contrast, during the second incubation period, $PGF_{2\alpha}$ significantly ($P < 0.01$) reduced the percent progesterone (respective values: 27.5 ± 1.7, 14.8 ± 2.4, 11.3 ± 2.4, 15.8 ± 1.9, and 16.8 ± 1.6%).

Finally, cAMP accumulation in the medium of the first 4-h incubation period was determined, as $PGF_{2\alpha}$ had been reported to rapidly inhibit LH-stimulated cAMP accumulation in rat CL [9, 12–14]. Luteinizing hormone increased the secreted cAMP from 0.21 ± 0.03 to 51.4 ± 5.5 pmol/well, and addition of 10 μM $PGF_{2\alpha}$ suppressed the LH-stimulated cAMP by 68% (to 16.4 ± 2.4 pmol/well). Because basal cAMP concentration was near the level of detection, no reliable conclusion could be made as to the effect of $PGF_{2\alpha}$ on basal cAMP.

Longer exposures to $PGF_{2\alpha}$ were examined next. Various concentrations of $PGF_{2\alpha}$ were added to cultures seeded on laminin, after an incubation of 7 days with and without PRL. Medium was collected for progesterone measurement after 22 h (Fig. 6), replaced by identical medium, and the incubation continued for an additional 23 h (six wells/treatment). In the absence of PRL, $PGF_{2\alpha}$ did not affect progesterone production at 22 h (Fig. 6) and at 45 h (values
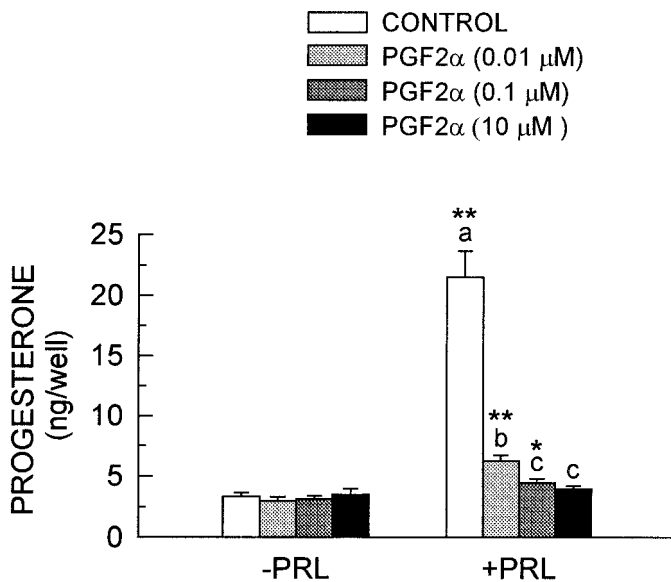
FIG. 6. Progesterone production by cells exposed to various concentrations of PGF$_{2\alpha}$ on Day 7 for 22 h in the presence and absence of PRL (200 ng/ml). Means $\pm$ SEM (n = 15 wells). Letters: Comparison among the various concentrations of PGF$_{2\alpha}$, within the same concentration of PRL (0 or 200 ng/ml). *$P < 0.005$, **$P < 0.001$ compared to the same concentration of PGF$_{2\alpha}$, but no PRL.

for 0, 0.01, 0.1, and 10 μM PGF$_{2\alpha}$: 3.10 $\pm$ 0.23, 3.79 $\pm$ 0.20, 2.89 $\pm$ 0.23, and 3.75 $\pm$ 0.21 ng/well, respectively). However, in PRL-treated cells, progesterone synthesis was greatly suppressed at all concentrations used, both at 22 h (Fig. 6) and at 45 h (respective values: 22.25 $\pm$ 2.84, 4.85 $\pm$ 0.22, 2.69 $\pm$ 0.28, and 3.64 $\pm$ 0.14 ng/well). This experiment was repeated, with identical findings. In later studies, a concentration of 100 nM PGF$_{2\alpha}$ was routinely used.

Figure 7 summarizes the results of 11 independent experiments, in which 100 nM PGF$_{2\alpha}$ was added to cultures 7–8 days after seeding on laminin or fibronectin, and the incubation continued for 45 h. In PRL-treated cells, production of both progestins was markedly suppressed by PGF$_{2\alpha}$, but inhibition of progesterone synthesis (by 68%) was significantly greater than inhibition of 20α-DHP (by 45%, $P < 0.001$). Thus, the percent progesterone out of total progestins was also significantly reduced by PGF$_{2\alpha}$. In contrast, in the absence of PRL, neither progesterone nor 20α-DHP were significantly affected by PGF$_{2\alpha}$; however, the percent progesterone was elevated slightly, but significantly (+18%).

Effect of PRL and PGF$_{2\alpha}$ on P450$_{scc}$ and 20α-HSD mRNAs

The described effects of PRL and PGF$_{2\alpha}$ on the percent progesterone out of total progestins are compatible with a decrease and an increase, respectively, in the activity of 20α-HSD. Thus, we examined the possibility that PRL suppressed 20α-HSD gene expression, and PGF$_{2\alpha}$ reversed this effect. Total RNA samples were prepared from cells incubated for 9 days with or without PRL, with PGF$_{2\alpha}$ added to half of the dishes for the last 45 h. Two sets of RNA samples were analyzed: the first was obtained by pooling RNA preparations from three independent experiments, and the second was derived from cultures of a single cell preparation. As shown in Figure 8, A–C, PRL lowered the concentration of 20α-HSD mRNA by 73%, and, in PRL-treated cells, PGF$_{2\alpha}$ doubled the amount of this message, compared
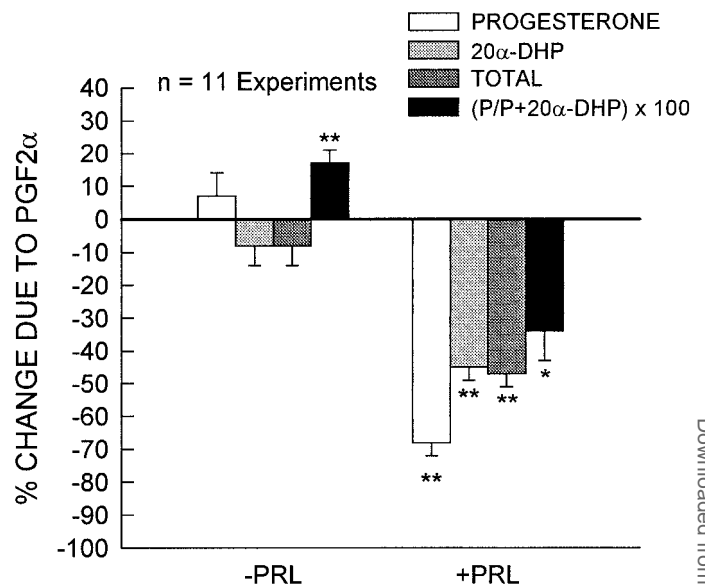

FIG. 7. Effect of PGF$_{2\alpha}$ (100 nM, 45 h) on progestin production in the presence and absence of PRL (200 ng/ml). The figure summarizes the results of 11 independent experiments. Calculations were as follows: 1) Progesterone and 20α-DHP were determined in the medium of each well, and total progestins, as well as the percent progesterone out of total progestins, were calculated. 2) Within a given experiment, averages were obtained for all the identically treated wells, for each of the four parameters. 3) Averages of the PGF$_{2\alpha}$-treated wells were expressed as a percentage of the respective control. 4) Means $\pm$ SEM (n = 11 experiments) of these values were then calculated for each parameter. 5) After subtraction of 100%, results are presented as the percent change (positive or negative) due to PGF$_{2\alpha}$. *$P < 0.005$, **$P < 0.001$ compared to no PGF$_{2\alpha}$.

to PRL alone. In the absence of PRL, PGF$_{2\alpha}$ had only a slight effect, if anything, on 20α-HSD mRNA.

In the same blots, P450$_{scc}$ mRNA was also estimated, as a reduction in this key steroidogenic enzyme may contribute to the inhibition by PGF$_{2\alpha}$ of total progestin production (Fig. 7). Figure 8, A, D, and E, shows that in PRL-treated cells PGF$_{2\alpha}$ reduced P450$_{scc}$ mRNA by nearly half, whereas in the absence of PRL, PGF$_{2\alpha}$ actually doubled the concentration of this message.

Effect of PGF$_{2\alpha}$ on DNA Amount and Fragmentation

In spite of the marked inhibition of steroidogenesis by PGF$_{2\alpha}$ (Fig. 7), the prostaglandin did not reduce the amount of DNA per well in these experiments. Expressed as a percentage of control, DNA values for PGF$_{2\alpha}$-treated cells in the absence and presence of PRL were, respectively, 103 $\pm$ 9% and 99 $\pm$ 6% (absolute values of the controls: 0.65 $\pm$ 0.14, and 0.67 $\pm$ 0.18 μg/well; n = 6 experiments, with cells seeded on laminin or fibronectin). Furthermore, Figure 9 demonstrates that such exposure to PGF$_{2\alpha}$ did not increase DNA fragmentation in PRL-treated cells.

Effect of PGF$_{2\alpha}$ on Phosphoinositide Metabolism

Because activation of the phosphoinositide-specific phospholipase C (PI-PLC) is considered the regular signal transduction mechanism of PGF$_{2\alpha}$ [34], we tested the effect of PGF$_{2\alpha}$ on PI-PLC activity in cultures incubated in the absence and presence of PRL. Cells were prelabeled with tritiated inositol, exposed to PGF$_{2\alpha}$ for 15 min, and inositol phosphates were determined as described in *Materials and Methods*.
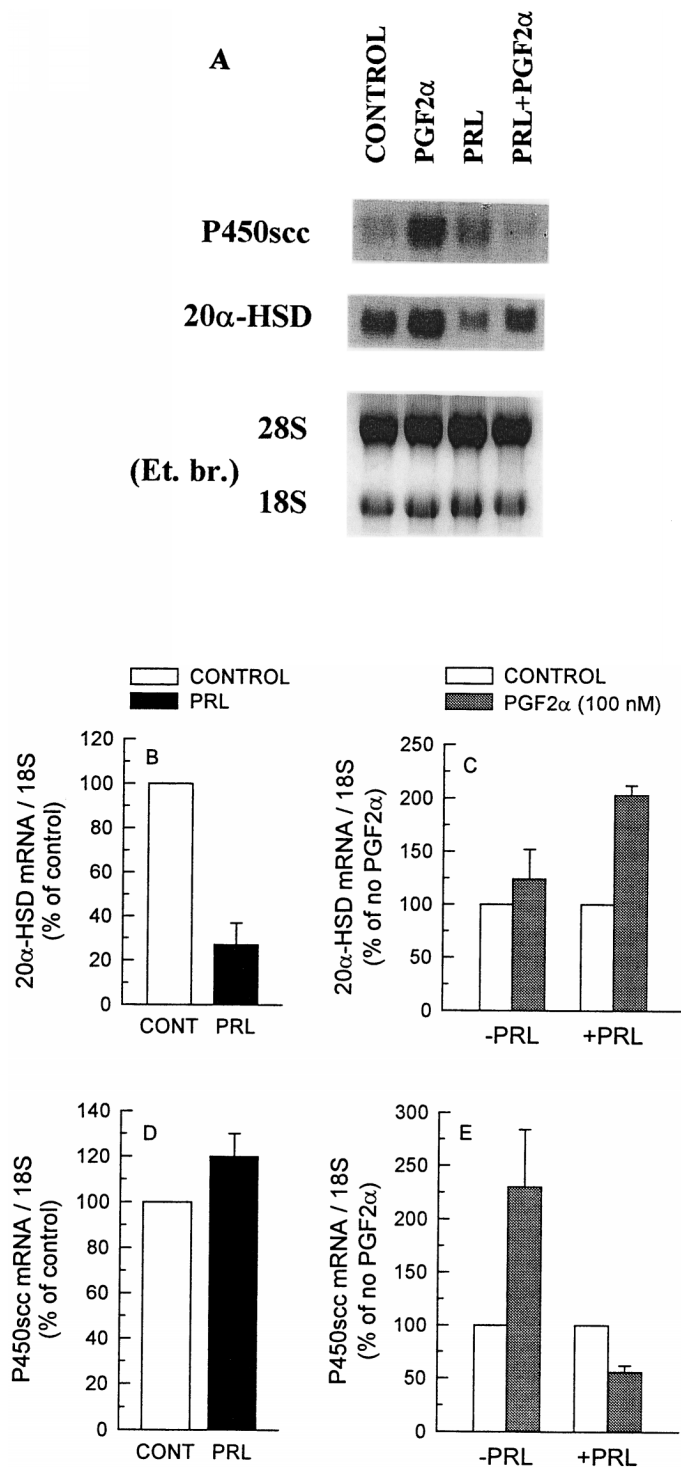
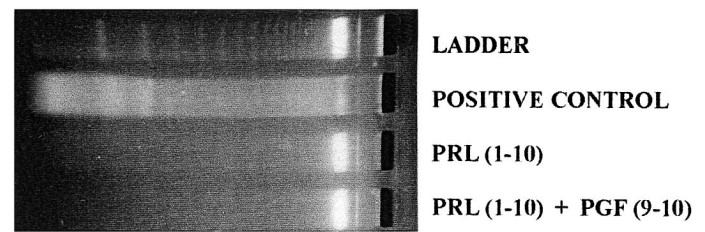Figure 10 shows that PGF$_{2\alpha}$ significantly stimulated the

FIG. 9. Agarose gel analysis of DNA from cells maintained with PRL (200 ng/ml) throughout the 10-day incubation, and treated with $PGF_{2\alpha}$ (100 nM, 45 h) or vehicle during the last 2 days. The positive control sample was apoptotic cells supplied with the kit and extracted by the same procedure applied to the luteal cells. Amount of DNA loaded: luteal samples, 5 μg; positive control, 8.4 μg.

activity of PI-PLC in the luteal cell cultures. The effect of $PGF_{2\alpha}$ in the absence of PRL was, if anything, greater than in its presence ($P = 0.051$ for the difference between the amounts of inositol phosphates in PRL-deprived and PRL-treated cultures after the addition of $PGF_{2\alpha}$).

DISCUSSION

Two main points of significance come out of the present investigation. First, the study characterized long-term rat luteal cell cultures that emerge as a suitable experimental model for studying in vitro the multistage process of $PGF_{2\alpha}$-induced luteolysis. Second, these cultures, in which PRL was not essential for either survival or steroidogenesis, enabled us to find that $PGF_{2\alpha}$ was luteolytic only in PRL-treated cells, whereas in the absence of PRL, $PGF_{2\alpha}$ exerted an alternative set of effects. Because PRL is essential for maturation of the rat CL, this finding may be relevant to the refractoriness of young CL to the luteolytic effect of
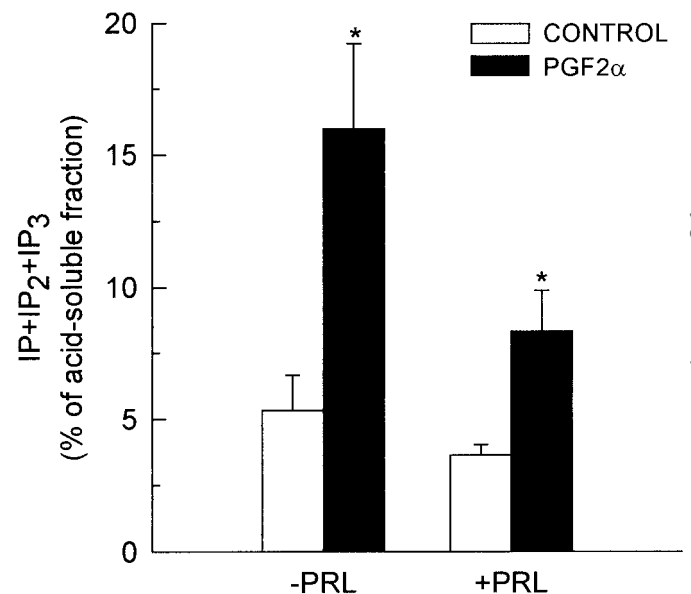

FIG. 8. Effects of PRL (200 ng/ml) and $PGF_{2\alpha}$ (100 nM, 45 h) on the concentrations of 20α-HSD and $P450_{scc}$ mRNAs. Twenty-microgram total RNA samples were subjected to Northern blot analysis. An autoradiogram of one experiment is presented in **A**; also shown are the rRNA bands in the respective gel, visualized by ethidium bromide and photographed in the negative mode. A second set of four RNA samples, obtained by combining RNA from three independent experiments, was similarly analyzed. **B–E**) Means ± range for these two blots. Messenger RNAs were quantified by phosphorimager, and values were normalized to the respective 18S rRNA, quantified by densitometry of the gel photograph. The effect of PRL on 20α-HSD (**B**) and $P450_{scc}$ (**D**) was calculated after normalizing the value obtained for PRL-treated cells to the control of the same blot. The effect of $PGF_{2\alpha}$ on 20α-HSD mRNA in the presence and absence of PRL is shown in **C**. Within each blot, values obtained for $PGF_{2\alpha}$-treated cells were normalized to the values of the appropriate control (i.e., same PRL but no $PGF_{2\alpha}$). The data for $P450_{scc}$ mRNA are similarly presented (**E**).

FIG. 10. Effect of $PGF_{2\alpha}$ (100 nM) on the production of [³H]inositol phosphates (IP + IP_2 + IP_3) in the presence and absence of PRL (200 ng/ml). Cells were prelabeled with [³H]inositol and after appropriate rinse were exposed to $PGF_{2\alpha}$ for 15 min in the presence of LiCl. Cells were extracted with 10% TCA, and the acid-soluble samples were fractionated on Dowex columns. The dpm in each fraction was expressed as a percentage of the total acid-soluble dpm in the same sample; the sum of the three inositol phosphates (IP + IP_2 + IP_3) was then calculated. Results are means ± SEM of six samples collected in three independent experiments. The mean ± SD of total acid-soluble dpm in the 24 samples was 119 592 ± 69 950. *$P < 0.02$ compared to same PRL but no $PGF_{2\alpha}$.

PGF$_{2\alpha}$, observed in vivo in many species, including the rat [15, 20, 21].

The cultures described here have several advantages. First, the incubation medium is entirely defined, and so are the proteins used to promote cell attachment, laminin and fibronectin. Another useful characteristic is the cultures' long-term stability, with and without added hormones. After cell attachment, total progestin production remained essentially constant for an additional week (Fig. 2; controls on Days 8 and 9 in the experiment of Fig. 6), suggesting no deterioration of steroidogenic cells during this period.

Laminin and fibronectin are components of natural luteal extracellular matrix [7, 35, 36]. In our cultures, laminin allowed for a larger number of attached cells compared to fibronectin, but we found relatively minor differences in cell function on the two matrices. In the study of Aten et al., the two proteins supported similarly the completion of luteinization [7]. In cultures of granulosa cells from rat preovulatory follicles [37], and cultures of mouse mammary epithelium [38], laminin was uniquely advantageous regarding cell survival and differentiation.

Several of the responses of our cultures to LH, PRL, and PGF$_{2\alpha}$ are recognized as physiological actions of these hormones in the rat CL; some of them, however, have been hitherto shown only in vivo. The effect of PRL to stimulate LH receptor expression [33] was implicated in the present study, although not directly shown: LH, either at 100 pg/ml added continuously (Fig. 2), or at 100 ng/ml added for 8 h (Fig. 3), affected steroidogenesis only in the presence of PRL. Such requirement of PRL was not apparent in freshly prepared luteal cell suspensions [9–11] or in short-term luteal cell cultures [22]. Thus, effects due to the in vivo exposure to endogenous PRL disappear slowly, and luteal cells truly devoid of PRL influence can be obtained only in long-term cultures.

The stimulation of steroidogenesis by LH, although significant, was rather modest (Fig. 4), in spite of the very high concomitant increase in cAMP. This response resembles the pattern observed in vivo more than that of luteal cell suspensions [4, 8–11] (see *Introduction*).

Another well-known action of PRL, corroborated here (Fig. 8), is the suppression of 20α-HSD activity [3, 4, 12, 15, 23], due to inhibition of 20α-HSD gene transcription [16–18]. However, the elevated percent progesterone out of total progestins in the presence of PRL (7–40% in the various experiments) was still much lower than the 65–75% recorded in the rats' plasma at the time they were killed. Sugino et al. [39] showed that progesterone and glucocorticoids inhibited 20α-HSD expression, apparently via the glucocorticoid receptor. In our cultures, dexamethasone (Dex) synergized with PRL in increasing the percent progesterone, whereas Dex alone had no effect (unpublished results).

The PRL-induced increase in the percent progesterone developed very slowly but was greatly accelerated by LH (100 pg/ml) (Fig. 2). Actions of LH that may be relevant are inhibition of 20α-HSD expression [17] and stimulation of expression of PRL receptors [40].

The most significant asset of the PRL-treated cultures is their suitability for exploring the longer-term events of PGF$_{2\alpha}$-initiated luteolysis. The twofold increase in 20α-HSD mRNA (Fig. 8), with corresponding reduction in the percent progesterone out of total progestins (Fig. 7), is, to our best knowledge, the first demonstration of 20α-HSD induction by PGF$_{2\alpha}$ in vitro, in rat luteal cells. The time course of this event—decreased percent progesterone at 8

h, but not at 4 h (text complementary to Fig. 5)—fits that of 20α-HSD induction in vivo [12, 15]. A very recent report by Stocco et al. [41] reveals the events underlying this delay: PGF$_{2\alpha}$ induces the expression of Nur77, and this transcription factor directly activates the 20α-HSD promoter.

The other important effect of PGF$_{2\alpha}$, namely, suppression of total progestin production, is complex, as indicated by previous studies. The inhibition of progestin synthesis, observed only 15 min after PGF$_{2\alpha}$ application in vivo [12] and in vitro [9, 14], presumably preceded any change in transcription. After an hour or more, PGF$_{2\alpha}$ reduced in vivo the expression of various steroidogenesis-associated genes: the steroidogenic acute regulatory protein (StAR, the effect on which included induction of the transcription factor DAX-1) [42, 43], sterol carrier protein-2, P450$_{scc}$, and 3β-HSD [44]. In another report, PGF$_{2\alpha}$ administration reduced after 1 h the amount of StAR protein but not StAR mRNA [45]. In luteal cell suspensions, induction by PGF$_{2\alpha}$ of heat-shock protein-70 mediated the inhibition of progesterone synthesis in cells treated with 8-bromo-cAMP and LH [46]. In the present study, inhibition of total progestin production was observed at all time points tested (4–45 h after PGF$_{2\alpha}$ addition). Cytochrome P450$_{scc}$ mRNA was estimated after a 45-h exposure to PGF$_{2\alpha}$, and a 45% inhibition was found (Fig. 8), similar to the reduction in total progestins at this time point (Fig. 7). Incidentally, PRL increased P450$_{scc}$ mRNA very slightly, if at all (Fig. 8). An augmentative effect of PRL on this message was demonstrated in luteinized rat granulosa cells [24]. While still ill-defined, the regulation of P450$_{scc}$ in rat CL was shown to be cAMP independent [24].

Inhibition of steroidogenesis by PGF$_{2\alpha}$ during the first 4 h was reversed by LH (Fig. 4) and so was the PRL-induced decrease in total progestins in cells seeded on fibronectin (Fig. 2B). The possibility that, in our cultures, both agents suppressed basal cAMP to below a critical level [12, 21, 22], while LH reversed this action, is at present highly speculative.

Prostaglandin F$_{2\alpha}$, although inhibiting progestin production, did not reduce the amount of DNA per well after 45 h nor did it cause DNA fragmentation at that time (Fig. 9) or after 4 days with PGF$_{2\alpha}$ (unpublished results). Thus, components of the extracellular matrix and/or medium may confer resistance to cell death [37], and/or additional factors (e.g., immune and endothelial cells, or soluble products thereof) are required for structural luteolysis [6, 47].

The most unexpected and intriguing finding of the present study is that PGF$_{2\alpha}$ had luteolytic effects only in PRL-treated cells. In the absence of PRL, progestin production was not inhibited by PGF$_{2\alpha}$ throughout the 45-h incubation (Figs. 3, 6, and 7). The concentration of P450$_{scc}$ mRNA was actually doubled with PGF$_{2\alpha}$ (Fig. 8), and the percent progesterone out of total progestins was slightly, but significantly, increased (Fig. 7). Thus, PGF$_{2\alpha}$ must have functional receptors in PRL-deprived cells.

Finally, PGF$_{2\alpha}$ stimulated the activity of PI-PLC both in the absence and the presence of PRL (Fig. 10). This finding indicates that activation of PI-PLC is not sufficient for inhibiting steroidogenesis. Some years ago we showed that PGF$_{2\alpha}$ stimulated PI-PLC activity in young (Day 2) CL, isolated from pseudopregnant rats but not in mature (Day 7) CL [48], although in the latter PGF$_{2\alpha}$ suppressed cAMP accumulation much more effectively [21]. Furthermore, increased concentrations of cytosolic calcium ions apparently

did not mediate the inhibitory effect of $PGF_{2\alpha}$ on cAMP [21].

In summary, we have established long-term, serum-free rat luteal cultures, in which PRL and LH are not survival factors. Some of PRL's well-known luteal effects are demonstrable in these cultures, and with the continuous presence of this hormone the cultures are a good model of mature CL, in that they respond to $PGF_{2\alpha}$ with the suppression of steroidogenesis and induction of 20α-HSD. In the absence of PRL, $PGF_{2\alpha}$ was not luteolytic but had a different set of effects. This experimental system will allow exploration of the signaling cascades of $PGF_{2\alpha}$ in the presence and absence of PRL and thus will better define their relevance to luteolysis.

ACKNOWLEDGMENTS

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