Prostaglandin $F_{2\alpha}$-Mediated Luteolytic and Luteotrophic Effects on the Human Granulosa-Luteal Cell

by

Jeffrey Eric Väänänen

B.Sc., Simon Fraser University, 1991
M.Sc., The University of British Columbia, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Obstetrics and Gynaecology,
Reproductive and Developmental Sciences Program)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March 1997

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of OBSTETRICS + GYNAECOLOGY

The University of British Columbia
Vancouver, Canada

Date 29 APRIL 1997

ABSTRACT

These studies examined the effects of prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) on progesterone and 17ß-estradiol (estradiol) production, as well as DNA and $PGF_{2\alpha}$-receptor ($PGF_{2\alpha}$-R) mRNA levels, in the human granulosa-luteal cell (GLC). Additionally, the interactions of $PGF_{2\alpha}$ with human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone (GnRH) and prostaglandin E_2 (PGE_2) were examined, with respect to progesterone and estradiol production. In one study, cells were collected from small (<12 mm) and large (>12 mm) follicles separately, permitting the examination of follicle size-dependent alterations in steroidogenesis. Pharmacological techniques were utilized to elucidate the signal transduction pathways involved in the anti-gonadotrophic effects of $PGF_{2\alpha}$. Moreover, these experiments were performed on GLCs cultured for one (D_1), eight (D_8) and/or twelve to fourteen days ($D_{12\text{-}14}$), in order to reveal culture time-dependent alterations in cellular response.

Briefly, GLCs collected from patients undergoing *in vitro* fertilization (IVF), were cultured for the time periods described above, followed by a 24 h treatment period. After the treatment period media were collected and assayed for progesterone and estradiol, while cells were extracted for DNA or total RNA.

It was found that human GLC responses to $PGF_{2\alpha}$ are culture time- and concentration-dependent, with $PGF_{2\alpha}$ being either luteolytic or luteotrophic, depending on culture and treatment conditions. Moreover, GLC responses to hCG and $PGF_{2\alpha}$ varied with follicle size, suggesting that these hormones' actions are targeted toward more mature follicles. Furthermore, GnRH potentiates the luteolytic effects of $PGF_{2\alpha}$, while it acts as a permissive factor for the luteotrophic effects. A complex interaction between $PGF_{2\alpha}$ and PGE_2 was also seen. The luteolytic effects of $PGF_{2\alpha}$ are mediated through a pertussis toxin-sensitive G-protein (possibly G_i, G_p or both). $PGF_{2\alpha}$ inhibits cholera toxin-, isoproterenol- and forskolin-, but not db-cAMP-stimulated progesterone production suggesting that this G-protein is exerting its actions on the adenylate cyclase pathway at the level of adenylate cyclase, but not distal to it. Additionally, $PGF_{2\alpha}$ is capable of autoregulating its receptor mRNA levels, and thus its ability to regulate steroidogenesis in the human GLC. Prostaglandin $F_{2\alpha}$-R mRNA levels were found to be inversely related to progesterone and estradiol production.

In conclusion, $PGF_{2\alpha}$ is a multi-functional hormone which acts through complex signal transduction pathways and interactions with confounding hormones, to exert both luteotrophic and luteolytic effects.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Category	**Abbreviation**	**Meaning**
Standard Abbreviations		
	ATP	Adenosine triphosphate
	AA	Arachidonic acid
	bp	Base pairs
	C	Celcius
	CA	California
	cAMP	Cyclic adenosine monophosphate
	cDNA	Complimentary DNA
	CL	Corpus luteum
	Cox-I	Cyclooxygenase I (constitutive)
	Cox-II	Cyclooxygenase II (inducible)
	CTX	Cholera Toxin
	2D	Two dimensional
	3D	Three dimensional
	D_1	Precultured for 1 day
	D_8	Precultured for 8 days
	D_{12-14}	Precultured for 12-14 fourteen days
	DAG	Diacylglyceriol or diglyceride
	db-cAMP	Dibutryl-cyclic-adenosine monophosphate
	ddH_2O	Double distilled water
	dCTP	Deoxycytosine-triphosphate
	dNTPs	Deoxynucleotide-triphosphate(s)
	DP	Prostaglandin D_2 receptor
	dpi	Dots per inch
	FSH	Follicle stimulating hormone
	DEPC	Diethylpyrocarbonate

DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide-triphosphates
DTT	Dithiothreitol
E_2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
EP_1	Prostaglandin E_2 receptor (isoform 1)
EP_3	Prostaglandin E_2 receptor (isoform 3)
Estradiol	17ß-estradiol
FBS	Foetal bovine serum
For	Forskolin
FP	Prostaglandin $F_{2\alpha}$ receptor
g	Grams
GLB	Gel loading buffer
GLC	Granulosa-luteal cell
GnRH	Gonadotrophin-releasing hormone
GTP	Guanosine triphosphate
G-protein	GTP dependent protein
G_α	G-protein alpha subunit(s)
$G_{\alpha s}$	G_α stimulatory
$G_{\alpha i1,2}$	G_α inhibitory (isoform 1 or 2)
$G_{\alpha i3}$	G_α inhibitory (isoform 3)
$G_{\alpha p}$	G_α placental
$G_{\alpha q}$	G_α placental (q isoform)
$G_{\alpha 11}$	G_α placental (11 isoform)
$G_{\alpha o}$	G_α olfactory
GRB	Gel running buffer
GTC	Guanosine thiocyanate Lysis buffer
h	Hours
hCG	Human chorionic gonadotrophin
Indo	Indomethacin

IP	Prostaglandin I_2 receptor
IP_3	Inositol trisphosphate
IsoP, or Iso	Isoproterenol
IU	International units
IVF	*In vitro* fertilization
Kd	Equilibrium dissociation constant
KDa	Kilodaltons
l	Liter
LH	Luteinizing hormone
LT	Leukotrienes
M199	Medium 199
ml	Milliliters
min	Minutes
MD	Maryland
mRNA	Messenger ribonucleic acid
MO	Missouri
m.w.	Molecular weight
M	Moles/Liter
$NADP^+$	Nicotinamine adenine dinucleotide phosphate
NADPH	Hydrogenated $NADP^+$
NH	New Hampshire
nM	Nanomolar
NY	New York
OD	Optical density
ON	Ontario
P_4	Progesterone
PA	Phosphatidic acid
PACAP	Pituitary adenylate cyclase activating polypeptide
PCR	Polymerase chain reaction

PBS	Phosphate-buffered saline
PG	Prostaglandin
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
$PGF_{2\alpha}$-R	Prostaglandin $F_{2\alpha}$ receptor
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H
PGI_2	Prostaglandin I_2
PI	Phosphoinositide
PKA	Protein kinase A
PKC	Protein kinase C
PKCi	Protein kinase C inhibitor Bisindolylmaleimide
PL	Prolactin
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
pM	Picomolar
pmole	Pico moles
PTX	Pertussis toxin
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT	Reverse transcription
s	Seconds
SCC	P450-side chain cleavage enzyme
SDS	Sodium dodecyl sulfate
Sigma	Sigma Chemical Company, St. Louis, MO.

SSC	Sodium chloride and sodium cytrate buffer
TBE	Tris borate EDTA
TRIS	Tris(hydroxymethyl)aminomethane
Tx	Thromboxanes
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
x	Times (or multiplied by)
x g	Times gravity
PQ	Quebec

Abbreviations Starting with Greek Characters

α	Alpha
ß	Beta
γ	Gamma
μM	Micromolar (10^{-6} molar)

ACKNOWLEDGMENTS

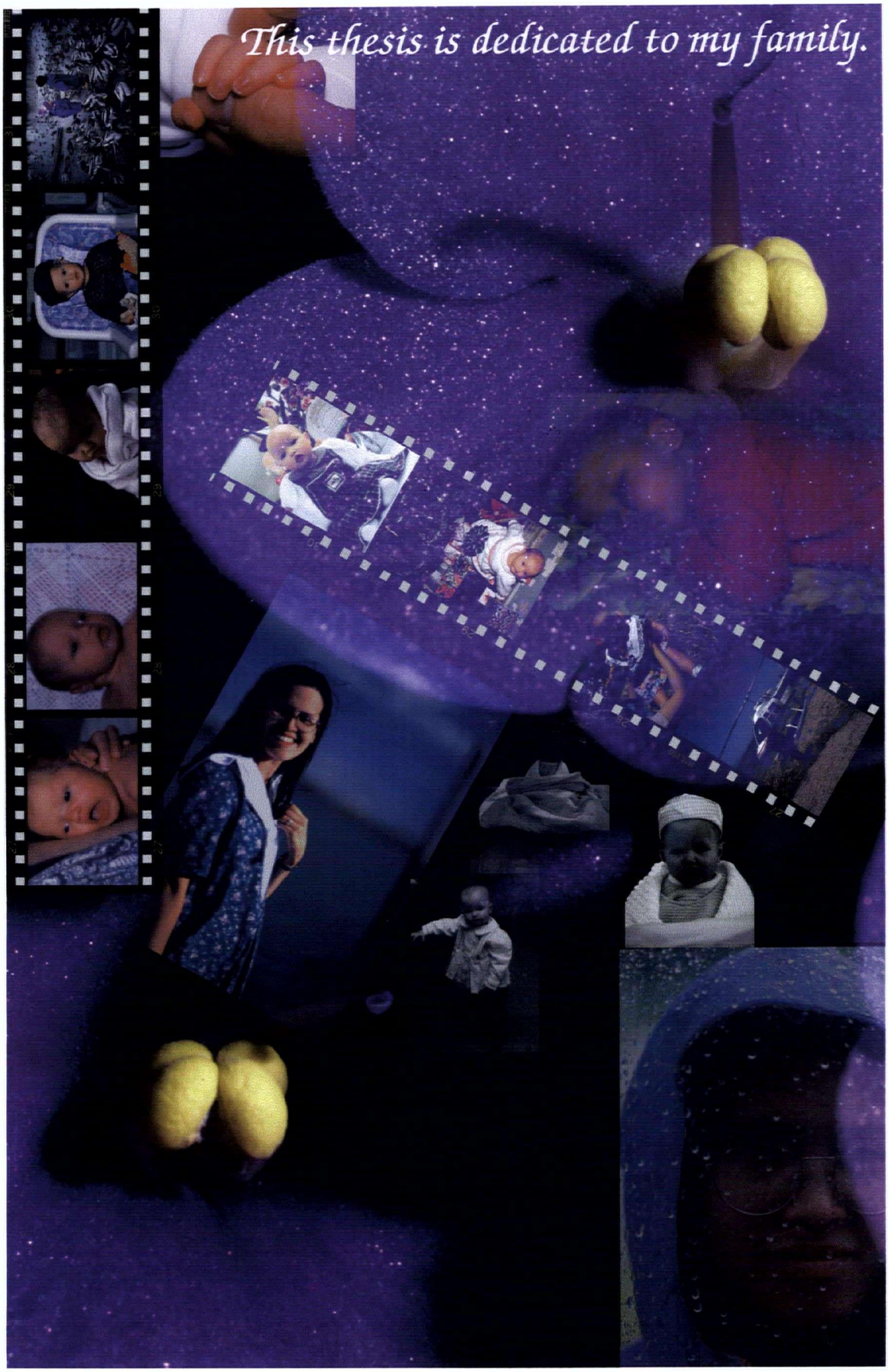
My gratitude to Dr. Leung for letting me explore my ideas while acting as a sounding board cannot be overstated. I would further like to thank him for allowing me the opportunity to gain experience by helping review papers, acting as chairperson for the departmental seminars and supervising three undergraduate students. This has truly been an educational experience. Many thanks to Dr. Ho Yuen for encouragement and instructive suggestions. I would also like to thank Dr. Auersperg for demonstrating so much enthusiasm after a good number of years of science. I hope that I may maintain such a passion for science. Dr. Rajamahendran's comments during my committee meetings have helped me tighten up my language. The chairman of my committee Dr. Lee helped strengthen this thesis by reinforcing my awareness of the some of the caveats of the human granulosa-luteal cell model. Additionally, Dr. Buchan, in the Department of Physiology, is largely responsible for the style and approach to science contained within this thesis, as her prolific advice during my Masters thesis still resides within my mind. I hope that she would be pleased with this manuscript.

Suzie, Brenda and Ivan deserve thanks for assistance about the lab, and for acting as guinea pigs during my attempts at being a teacher. I hope that they took away as much as they gave. Pearly has provided some good advice, and a great deal of entertaining conversation. I appreciate Pearly's willingness to process cells on alternate days with me.

The IVF staff have been incredibly supportive, and have provided me with cells literally every day that it was possible to provide them. Special words of praise should be reserved for Cindy and Pathma for their attention to detail and enthusiasm for research.

I would like to thank the folks at the Center for Evaluation Sciences for the two talks on statistics that they gave during my tenure in the department, and for the free consultations reguarding my project. It was reassuring to talk to an expert in that discipline.

My friend Janine Senz was always willing to answer the phone at two in the morning and provide molecular biology tips– for this she must be thanked. Ted Urbanek, who provided me with diversions when I couldn't continue to work also deserves comment. Having worked for countless years as a journalist, both independently and for the Canadian Broadcasting Corporation (CBC), my uncle Garth Cochran, provided an endless source of suggestions, corrections and typesetting. My parents have instilled in me, determination and an interest in science, the two most important driving forces in the underlying studies. Without Céline, my tag team partner, I could not have finished this thesis. Céline has been a wonderful friend, playmate, helper, wife and mother to my child(ren). If everyone was as silently confident and wise as Céline the world would be a much more generous and peaceful place. Her comfort with herself is something that I shall forever envy. At our wedding, Celine's family showed me kindness and acceptance that I have never known. Their support has been crucial in this last three years.


This thesis is dedicated to my family.

I – BACKGROUND

A. The Classical Neuro-Endocrine Pathway of Gonadal Regulation

Classically, regulation of ovarian steroidogenesis was seen as a purely hypothalamo-pituitary axis phenomena (Fig. 1). The classical neuro-endocrine pathway acts as follows. Various inputs such as corticotrophin releasing hormone, dopamine, endorphin, estradiol, norepinephrine, pheromones, serotonin and the light/dark cycle are integrated in the arcuate and preoptic nuclei [Advis et al., 1978; Balthazart et al., 1981; Donham et al., 1993; Dufour et al., 1988; Laatikainen, 1991; Rotsztejn et al., 1976; Sawyer, 1975; Yen et al., 1977]. These influences regulate the secretion of gonadotrophin-releasing hormone (GnRH) from neuron-like cells, originating in these nuclei, and terminating in the anterior pituitary. Gonadotrophin-releasing hormone is a decapeptide that is clipped from a larger propeptide [Hsueh et al., 1983; Nillius et al., 1974]. Through a receptor-dependent mechanism, GnRH acts on gonadotrophs to stimulate the release of the gonadotrophins, follicle stimulating hormone (FSH) and lutenizing hormone (LH) [Baldwin et al., 1984; Joshi et al., 1993; Rommler et al., 1979]. Follicle stimulating hormone and LH are collected by the portal system of the anterior pituitary and distributed, via the efferent veins, into the general circulation where they eventually reach their target the ovaries [Sawyer, 1975].

Lutenizing hormone is secreted in pulses, with the period between peaks being 1 to 7 hours depending on the phase of the menstrual cycle [Filicori et al., 1979]. The pulsatile nature of LH release is probably due to pulsatile GnRH secretion or GnRH-receptor number fluctuations, rather than gonadal feedback [Baldwin et al., 1984; Inaudi et al, 1992; Schuiling and Gnodde, 1976]. During the follicular phase estradiol levels increase in response to FSH. When the developing follicle is fully mature, the estradiol levels reach a threshold which initiates an LH peak and triggers ovulation.

Follicle stimulating hormone is released from a single pool, in a pulsatile manner, with a lower amplitude than LH [Filicori et al., 1979]. The release of FSH is less sensitive to GnRH than LH [Hall et al., 1992], and can further be regulated by estradiol.

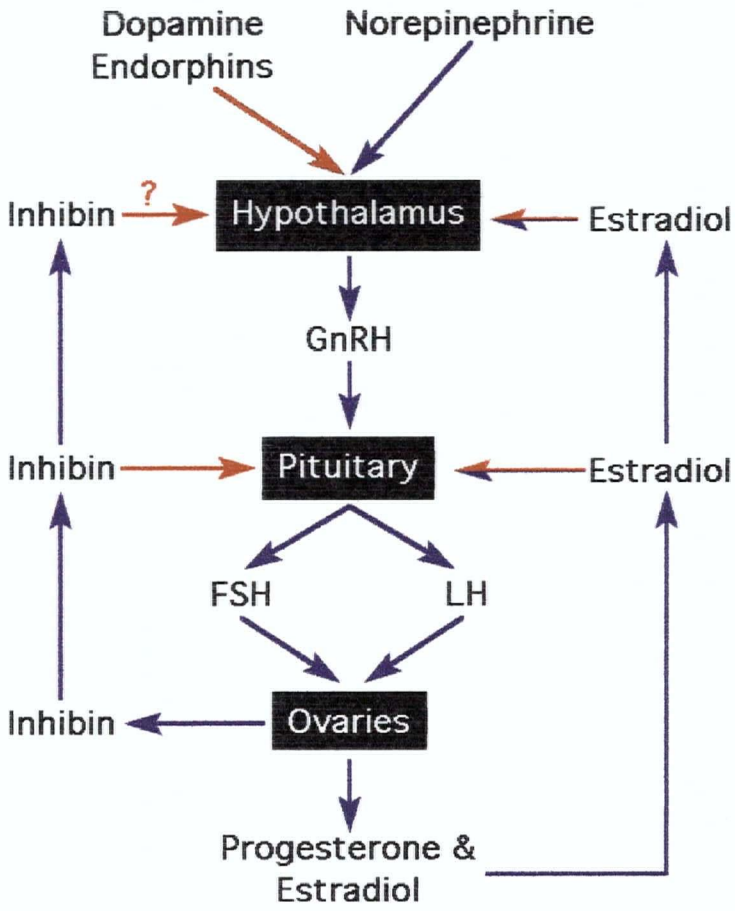


Figure 1. The hypothalamopituitary axis. Various stimulatory (⟶) and inhibitory (⟶) neural inputs regulate the secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus. GnRH in turn stimulates the production and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. Circulating FSH and LH stimulate progesterone, estradiol and inhibin production from the ovaries. Inhibin exerts negative feedback on the pituitary and possibly the hypothalamus. Additionally, follistatin (an ovarian product) inhibits the actions of inhibin (not diagrammed). Estradiol can positively or negatively feedback on the pituitary and hypothalamus depending on temporal and concentration conditions.

Both FSH and LH are glycoproteins which share a common α-subunit (m.w. 14,000; 96 AA). Additionally, FSH and LH each have a unique ß-subunit, which is noncovalently linked to the α-subunit [Combarnous, 1988; Gray , 1988; Ryan et al., 1987; Wierman et al., 1988]. These peptides possess carbohydrate moieties which account for 15 percent of their weight, and are involved in receptor binding [Combarnous, 1988; Gray , 1988; Ryan et al., 1987; Wierman et al., 1988].

Once FSH and LH reach their primary target in the female– the ovaries their actions diverge. In the granulosa cell FSH is responsible for stimulating mitosis, aromatase activity and inducing LH-receptor expression and membrane presentation. These actions serve to ripen or prepare the developing follicle for ovulation. This FSH-induced increase in LH-receptors primes the granulosa cell for the LH surge just prior to ovulation. Lutenizing hormones primary action on the granulosa cell is an increase in progesterone synthesis. Furthermore, in the theca cell LH promotes mitosis and progesterone and androgen synthesis. With the granulosa and theca cells working in concert, estradiol is released into the intracellular space where it feeds back on both cell types. Estradiol promotes FSH-receptor and estradiol-receptor expression on the granulosa cell, and LH-receptor expression on the theca cell, further enhancing the actions of these hormones. Additionally, estradiol feeds back on the anterior pituitary to increase GnRH secretion as well as the pituitary response to it [Burger, 1981]. The increase in GnRH and the pituitary's sensitivity to it, increases LH secretion and decreases FSH secretion. This feedback further promotes the ripening of the follicle in preparation for ovulation. Ovulation is induced by LH in concert with numerous peptides, steroids, prostaglandins, leukotrienes and neurotransmitters, including but not limited to: collagenase, epidermal growth factor, relaxin, GnRH, vasoactive intestinal polypeptide, progesterone, prostaglandin $F_{2\alpha}$, prostaglandin E_2 and possibly prostaglandin I_2. For further information on the mechanisms of ovulation, see the following reviews: Channing et al., 1980; Haour and Lang, 1978; Leung and Steele, 1992; Suzuki and Takahashi, 1974; Turgeon, 1980; Wu and Prazak, 1974; Yen, 1977.

B. Pregnancy

Following ovulation, the ovum is transported down the fallopian tubes where fertilization occurs, usually within 12 to 24 hours post-ovulation. If fertilization has been successful the zygote will pass through the fallopian tubes (2-3 days) and implant in the uterus (approx. 3 more days). The key hormones in promoting and maintaining pregnancy are estradiol and progesterone (reviewed below, p. 4). The post-ovulatory follicle differentiates into the corpus luteum following the ovulatory phase. Granulosa cells differentiate into luteal cells account for about 80 percent of the corpus luteum (large luteal cells), with the remainder of luteal cells being derived from the theca interna (small luteal cells). The corpus luteum is the primary source of sex steroids during the luteal phase. Moreover, if fertilization occurs, the luteal phase is maintained beyond its 14 day lifespan by conceptus and/or placental derived hCG, which stimulates steroidogenesis through a cAMP dependent mechanism (Fig. 2). The corpus luteum is maintained until placental derived progesterone levels are adequate to maintain pregnancy, after which time it regresses. The regressed corpus luteum either in pregnancy or in the menstrual cycle is called the corpus albicans.

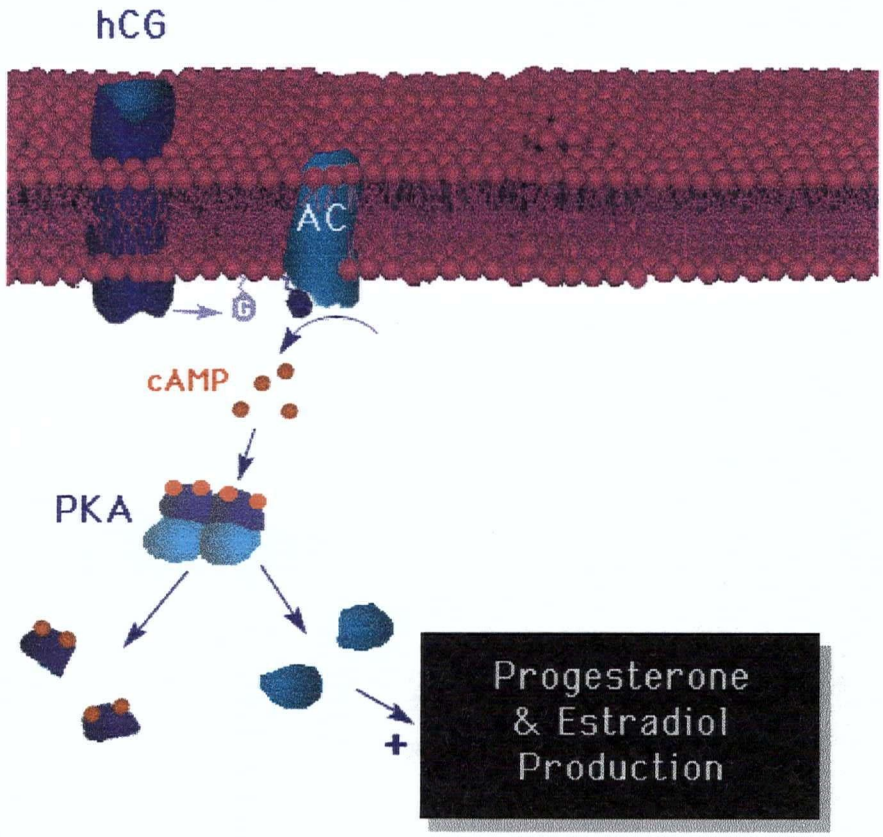


Figure 2. Model of signal transduction pathway for human chorionic gonadotrophin (hCG) stimulated steroidogenesis, in human luteal cells. G - stimulatory G-protein; AC - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A.

C. The Sex Steroids

The Progestins and Estrogens

The key sex steroid hormones are the progestins and the estrogens. Progestins are known as the pro-gestational hormones for their ability to maintain, prepare for and promote pregnancy. The progestins include progesterone, 17α-OH-progesterone and 20α-OH-progesterone, of which progesterone is the most potent. The estrogens are responsible for the secondary sex characteristics of the female, follicle maturation, and are behavioural modifiers in animals and possibly humans. In animals, the estrogens are reported to promote estrous behaviour (or mating behaviour), hence the name estrogen (a derivative of 'estrous-genic'). The estrogens include 17ß-estradiol (commonly known as estradiol or E_2) and estrone, of which estradiol is the most potent.

The Synthesis of Progesterone and Estradiol

The sex steroids are synthesized in the ovarian granulosa, luteal and thecal cells where they are known to have paracrine and/or autocrine actions in addition to their peripheral endocrine effects. Progesterone and estradiol are synthesized from cholesterol, which may be obtained from dietary sources or synthesized from two acetyl-CoA molecules, by a series of enzymatic reactions [Stryder 1988; Schroepfer 1982; Fielding 1985; Nebert and Gonzales 1987; Granner 1988]. The side chain of cholesterol is cleaved by P450-side chain cleavage enzyme (P450-SCC) or 20,22-desmolase to produce pregnenolone (Fig. 3). Pregnenolone may then be converted to progesterone by a complex of 3ß-ol-dehydrogenase and $^{\Delta 4,\ 5}$-isomerase. Through a series of enzymatic reactions, progesterone or pregnenolone may be converted to estradiol. One of the key enzymes in this conversion is aromatase. Aromatase and P450-SCC are highly regulated enzymes, as discussed below. For a more complete description of these synthetic pathways, please refer to Figure 3.

The Two–Cell Model of Steroid Biosynthesis

In the human ovary it requires the co-operation of two different cell types, the theca interna cell and the granulosa cell, to produce estrogen [Moon et al., 1978; Moon et al., 1981; Tsang et al., 1982; Moon and Duleba 1982; Takahashi et al., 1984]. This two–cell model of steroidogenesis is depicted in Figure 4. Briefly, LH stimulates cAMP production in the theca

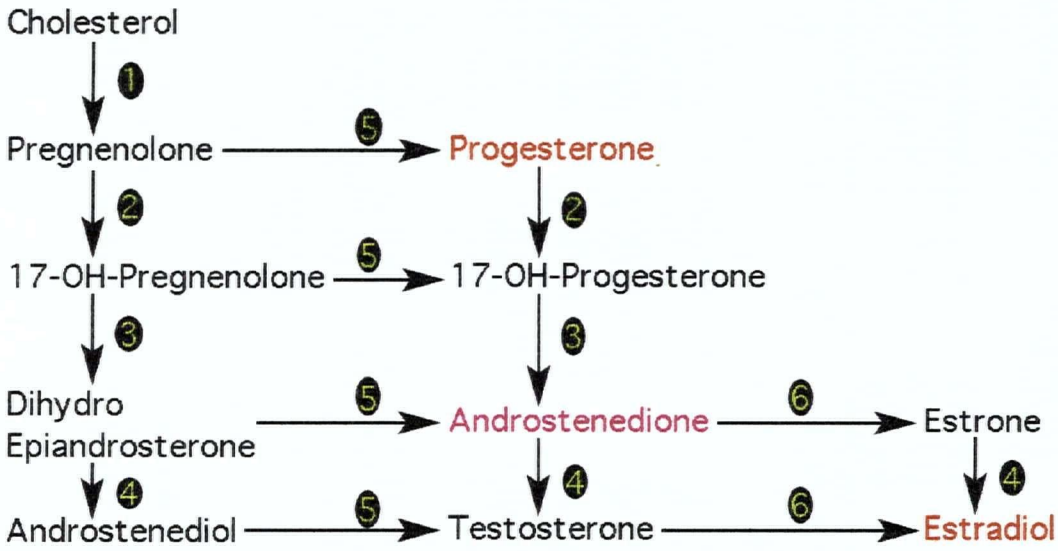


Figure 3. The synthetic pathway of the female sex steroids progesterone and estradiol, from cholesterol. The enzymes involved in sex steroidogenesis include: 1) P450 side chain cleavage enzyme or 20, 22-desmolase, 2) 17-hydroxylase, 3) 17, 20-desmolase, 4) 17ß-OH-steroid dehydrogenase, 5) 3ß-ol-dehydrogenase and $^{\Delta 4, 5}$-isomerase, and 6) aromatase. In the studies presented herewithin, androstenedione is added to the culture medium to provide an aromatizable substrate for the production of estradiol.

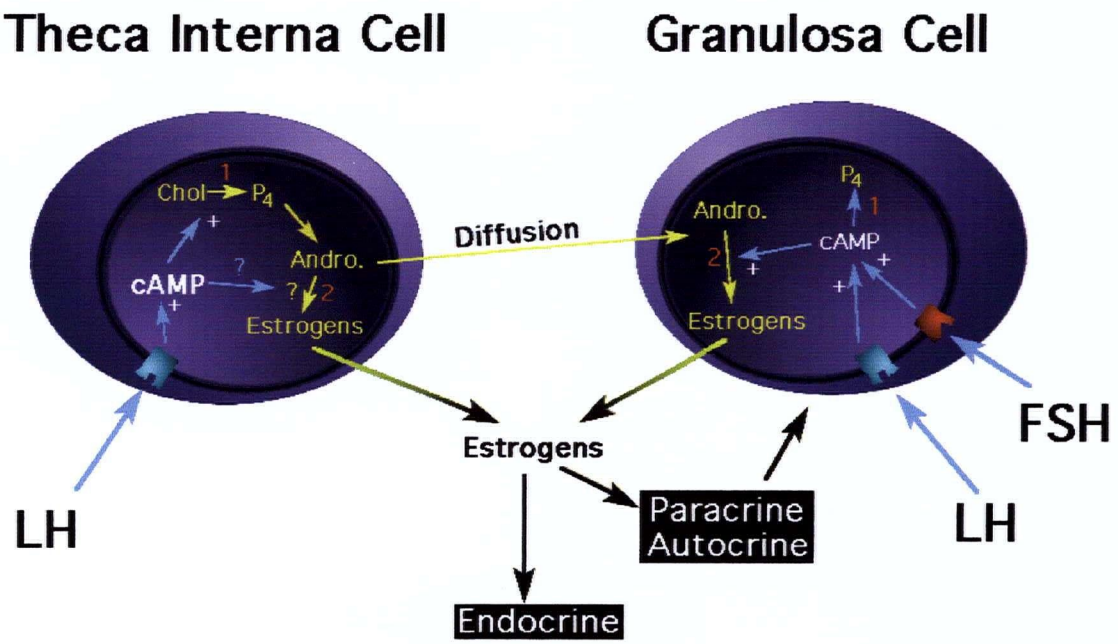


Figure 4. The two cell model of steroidogenesis in the human ovary. Luteinizing hormone (LH) stimulates cAMP production in the theca interna and granulosa cells, while follicle stimulating hormone (FSH) stimulates cAMP production in the granulosa cell. Progesterone (P_4) is produced via a cAMP-mediated increase in desmolase (1) activity, in both cell types. However only the theca interna cells are able to convert progesterone to aromatizable androgens. In order for the granulosa cell to produce estrogens an exogenous source of aromatizable androgens is necessary. These exogenous androgens are provided through diffusion from the theca interna cells to the granulosa cells. The conversion of androgens to estrogens is achieved by cAMP-mediated increase in aromatase (2) activity.

interna and granulosa cells, while FSH stimulates cAMP production in the granulosa cell. Progesterone is produced in both cell types via a cAMP-mediated increase in desmolase activity. However, only the theca interna cells are able to convert progesterone to aromatizable androgens. In order for the granulosa cell to produce estrogens, an exogenous source of aromatizable androgens is necessary. These exogenous androgens are provided through diffusion from the theca interna cells to the granulosa cells. In an *in vitro* culture system, it is necessary to provide granulosa cells with exogenous androgens (usually androstenedione or testosterone), if one wishes to measure estradiol production in response to stimuli. The conversion of androgens to estrogens is achieved by cAMP-mediated increase in aromatase activity.

Sex Steroid Receptors

Following synthesis, the sex steroids have local effects within the ovary, as well as endocrine effects throughout the body and hypothalmo-pituitary axis [Goebelsmann 1979; McCarty et al., 1983; McNatty et al., 1979a and b; Schroepfer 1982; Rasmussen and Yen 1983; Nebert and Gonzalez 1987]. The majority of progestins and estrogens circulate bound to binding proteins including albumin, cortisol binding protein and sex steroid binding protein. Only one to two percent of these steroids circulate in their free form.

Due to the hydrophobic nature of steroid hormones, they readily pass though cellular membranes, both from their sites of production and into their sites of action. Thus, these hormones do not have membrane receptors. This has the advantage that it eliminates the need for a secondary messenger system. Steroid receptors belong to a super-family of receptors which also include the thyroid hormone, retinoic acid and vitamin D receptors [McCarty et al., 1983]. The progestin and estrogen receptors each possess a DNA binding domain and a ligand binding domain. Following binding of the steroid to its receptor, the receptor-steroid complex attaches to its DNA acceptor site. This complex forms a site for the binding of RNA polymerase to the chromosome, and results in the production of RNA transcripts and their associated proteins. These *de novo* proteins are responsible for steroid-mediated cellular actions.

Sex Steroid Sites of Action

The regulation of the human menstrual cycle, conception and pregnancy by progesterone and estradiol is a body-wide process involving the brain, pituitary, ovary, uterus, fallopian tubes, vagina, breasts and other tissues. The following is a brief review of the effects of progesterone and estradiol on these tissues. As the hypothalamopituitary-gonadal axis has already been reviewed, this section will not discuss them further [see Mahesh 1985; Franz 1988; Tonetta 1989; and Genuth 1988 for further review]. Likewise, there are too many sex steroid-dependent functions throughout the body to discuss them all in the context of this thesis.

1. The Fallopian Tubes

Following ovulation estradiol assists in the capture and transportation of the ovum down the fallopian tube [Spilman and Harper 1975; Genuth 1988; Janzen 1995]. Estradiol is responsible for the widening and undulatory movement of the fimbria which assists in catching the ovum and directing it into the fallopian tube. The number of cilia on the surface of fallopian tube epithelial cells is increased by estradiol. Once in one of the fallopian tube, the ovum is transported toward the uterus by an estradiol-dependent beating of epithelial cilia and fallopian tube contractions. During the luteal phase, progesterone maximizes the cilliary beating and increases nutrient secretion into the lumen of the fallopian tubes. These nutrients may help to maintain the viability of both the ovum, sperm and eventually the zygote if fertilization occurs.

2. The Uterus

Elevated estradiol levels during the follicular phase are responsible for an increase in endometrial thickness (3- to 5-fold), and elevated levels of watery, strand-like mucus [Bazer et al., 1979; Janne 1981]. The increase in endometrial thickness may be in preparation for implantation, and establishes a nutritive base for the new conceptus. Elevated levels of fluid, strand-like mucous create channels to allow sperm to pass freely through the cervix into the uterus. Thus, estradiol is responsible for creating a uterine environment conducive to fertilization and implantion.

On the contrary, elevated progesterone levels reduce mitotic activity and the proliferation of the endometrium, although it is responsible for maintenance of the decidual lining [Genuth 1988]. Progesterone increases glycogen accumulation in vacuoles at the base of endometrial cells, and stimulates the movement of these vacuoles towards the lumen during the luteal phase. These glycogen stores provide an energy rich environment for the zygote within the lumen during implantation. Progesterone reduces the levels of mucus, and changes the mucus from fluid to viscous. These changes assist in implantation of the conceptus within the uterus.

3. The Vagina

Estradiol assists in successful copulation by improving vaginal conditions such as increasing mucous secretions, mucus fluidity, epithelial thickness (protective), vaginal plasticity and external genitalia size [Genuth 1988]. Following the ovulatory phase when it would be less appropriate for copulation to occur, progesterone reduces secretions, secretion fluidity and the numbers of cornified cells [Genuth 1988].

4. The Breasts

In preparation for pregnancy, estradiol promotes the development of the breasts by increasing fat deposits (i.e. energy stores) and the number of lobules [Mauvais et al., 1986; Mauvais et al., 1986; Mauvais et al., 1987]. These changes are in concert with progesterone-mediated alveoli formation. Thus, should pregnancy occur, the breasts will be partially prepared to fulfill their role as a primary nutrient dispensary for the neonate.

5. Other Progesterone-Dependent Actions

A number of other tissues are dependent on the sex steroids for their reproductive functions [Siiteri 1987]. Progesterone acts as a primary substrate for the production of cortisol and aldosterone by the foetal adrenal gland. Additionally, the crucial inhibition of the maternal immune response to foetal antigens is regulated by progesterone [Genuth 1988]. Progesterone also suppresses uterine contractions and expulsion of the foetus from the uterus. Progesterone also acts as a pyrogen, through a thyroid gland mediated increase in metabolism, which elevates body temperature. Behavioural effects have also been reported [Barfield et al., 1984].

D. The Eicosanoids

Prostaglandins, Thromboxanes and Leukotrienes

Membrane phospholipids can be metabolized into a class of hormones called the eicosanoids [Smith 1985; Mayes 1988]. The eicosanoids are further broken up into one of three sub-families, including the prostaglandins (PG), thromboxanes (Tx) and leukotrienes (LT). These hormone sub-families contain a number of hormones each designated by a letter such as A, B, C, et cetera. This character is further followed by a subscript number indicating the number of double bonds contained in the hormone. Furthermore, there are three groups within each of these three eicosanoid sub-families: those with one, two or three double bonds (Table 1). For example, the double bonded form of prostaglandin E is abbreviated PGE_2.

Phospholipases and Arachidonic Acid

The main precursor to eicosanoid synthesis is a twenty carbon, four double-bond fatty acid called 5, 8, 11, 14-eicosatetraenoic acid, commonly known as arachidonic acid (AA). The primary enzyme responsible for the production of AA is phospholipase A_2 (PLA_2), although a number of other lipases are capable of producing AA from glycerophospholipid precursors [Waite 1985; Dennis 1983].

Phospholipase A_2 is a hydrophobic, membrane-bound esterase which is active at the water-lipid interphase [Waite 1985]. The family of phospholipases consists of at least five members including phospholipase A_1, A_2 (B), C and D, each of which cleaves phospholipids at a unique site (Fig. 5) [Mayes 1988]. Normal saturation kinetics do not apply to membrane-bound phospholipases, as they do to soluble esterases. Compared to soluble esterases, phospholipases are exposed to extremely high concentrations of substrate molecules (phosphlipids), which are pre-oriented toward the catalytic site due to their polarity [Waite 1985]. Moreover, phospholipase enzyme products are hydrophilic, a property which enhances their diffusion away from the enzyme and the hydrophobic membrane, thus reducing product inhibition of substrate catalysis. Phospholipases can be greater than 1000 times more active than soluble esterases, due to their aforementioned properties.

Table 1. The Eicosanoid Superfamily of Hormones.

	Group I			**Group II**			**Group III**		
Fatty Acid Precursor	**8, 11, 14-Eicosatrienoate**			**Arachidonic acid**			**5,8,11,14,17-Eicosapentaenoate**		
Enzyme*	**COX**		**Lipox**	**COX**		**Lipox**	**COX**		**Lipox**
Eicosanoid	PGE_1	TxA_1	LTA_3	PGD_2	TxA_2	LTA_4	PGD_3	TxA_3	LTA_5
	PGF_1		LTC_3	PGE_2		LTB_4	PGE_3		LTB_5
			LTD_3	$PGF_{2\alpha}$		LTC_4	PGF_3		LTC_5
				PGI_2		LTD_4			
						LTE_2			

PG - Prostaglandin; Tx - thromboxanes; LT - leukotrienes. Group I, II, and III possess 1, 2 and 3 double bonds, respectively. * the key enzyme responsible for metabolism from the above fatty acid precursor, including: cyclooxygenase (COX) and lipoxygenase (Lipox).

In a number of systems, the reported pathway for the activation of PLA_2 involves a receptor mediated rise in intracellular calcium, which activates phospholipase C (PLC). Phosphatidyl inositol (PI) cleavage by PLC produces diacylglyceride which can either be converted directly to AA by glyceride lipase(s), or may stimulate diacylglyceride (diglyceride) dependent-protein kinase C (PKC) which in turn activates PLA_2, via removal of tonic inhibition by a protein inhibitor (Fig. 6) [Waite 1985]. Other factors influencing the activation of PLA_2 include membrane charge (and associated enzyme pH), density of phospholipids and membrane fluidity. Factors which affect these three parameters will alter PLA_2 activity [Waite 1985] and AA production. Finally, anti-inflammatory corticoids can block the PLA_2 activity.

Eicosanoid Production from Arachidonic Acid

Two isoforms of cyclooxygenase (COX-I, constitutive and COX-II, inducible) are capable of converting arachidonic acid to prostaglandin G_2 (PGG_2; Fig. 7). Cyclooxygenase I and II are selectively inhibitable by numerous anti-inflammatory agents. Inhibitors of COX-I include acetylsalicyclate and indomethacin [Vane 1971; Roth and Siok 1978], while dexamethasone and other modern nonsteroidal anti-inflammatory agents inhibit COX-II [McCarthy 1995]. Hydroperoxidase converts PGG_2 to prostaglandin H_2 (PGH_2), the precursor to group II or double bonded prostaglandins and thromboxanes. PGH-PGE isomerase converts PGH_2 to PGE_2, which can be further converted $PGF_{2\alpha}$ by E-2-9 ketoreductase. Theoretically, $PGF_{2\alpha}$ could be produced directly from PGH_2 by a reductase, although this pathway has not been demonstrated [Smith 1985].

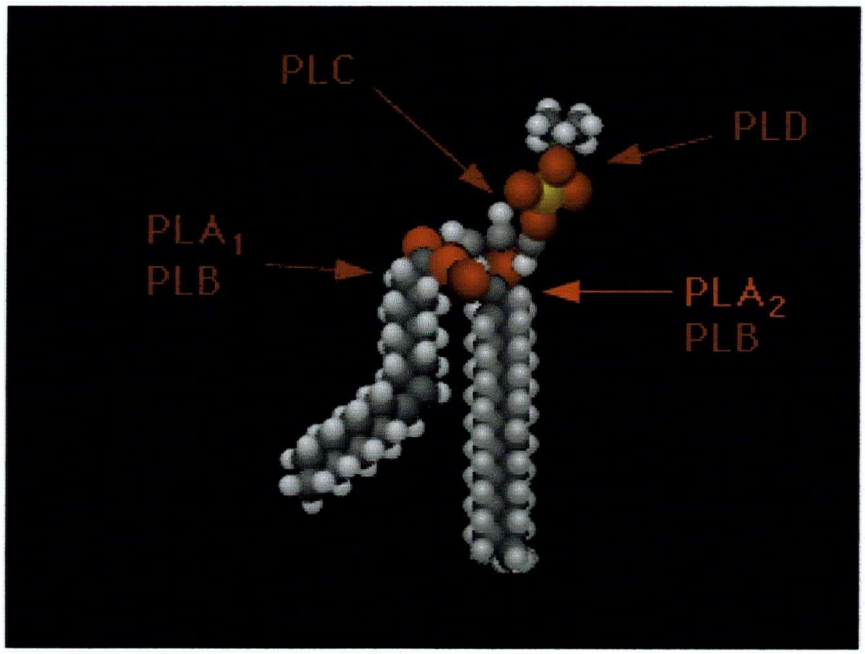


Figure 5. Phospholipase cleavage (hydrolysis) sites on phospholipids. Phospholipases are capable of hydrolysing the number one acyl bond, number two acyl bond, glycerophosphate bond or the base group. The number one acyl bond is hydrolysed by phospholipase A_1 (PLA_1) or PLB , while the number two acyl bond is hydrolysed by phospholipase A_2 or B (PLA_2 or PLB). The phosphodiesterases, phospholipase C (PLC) and phospholipase D (PLD) hydrolyse the glycerophosphate bond and base group, respectively.

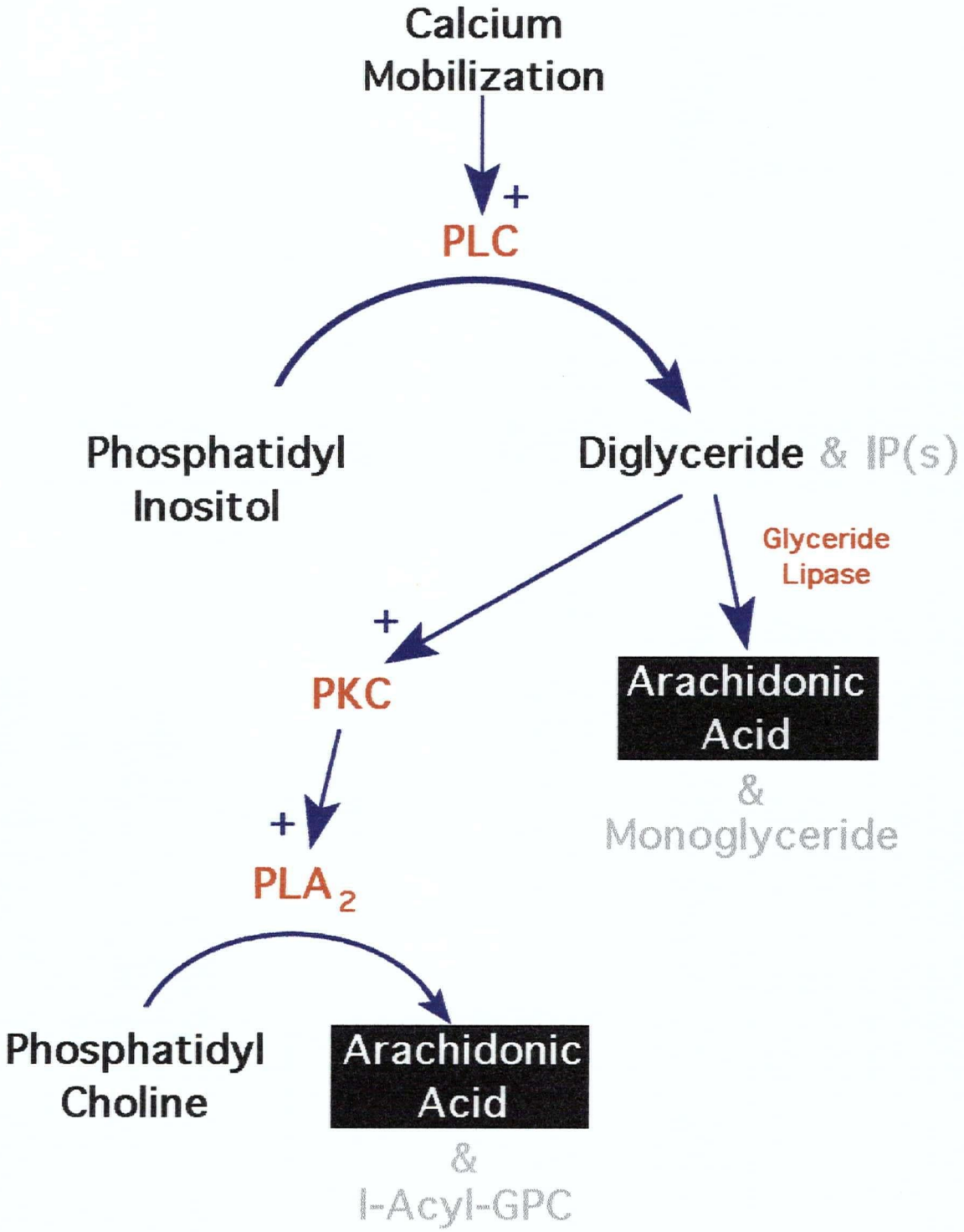


Figure 6. Arachidonic acid production in a model system. This model is based on research performed on platelets. In platelets calcium is mobilized via some external stimuli. Elevated calcium levels activate phospholipase C (PLC) which liberates diglyceride from phosphatidyl inositol. Glyceride lipase can convert diglyceride to arachidonic acid directly. Alternately, diglyceride may activate phospholipase A_2 (PLA_2), which converts phosphatidyl choline to arachidonic acid.

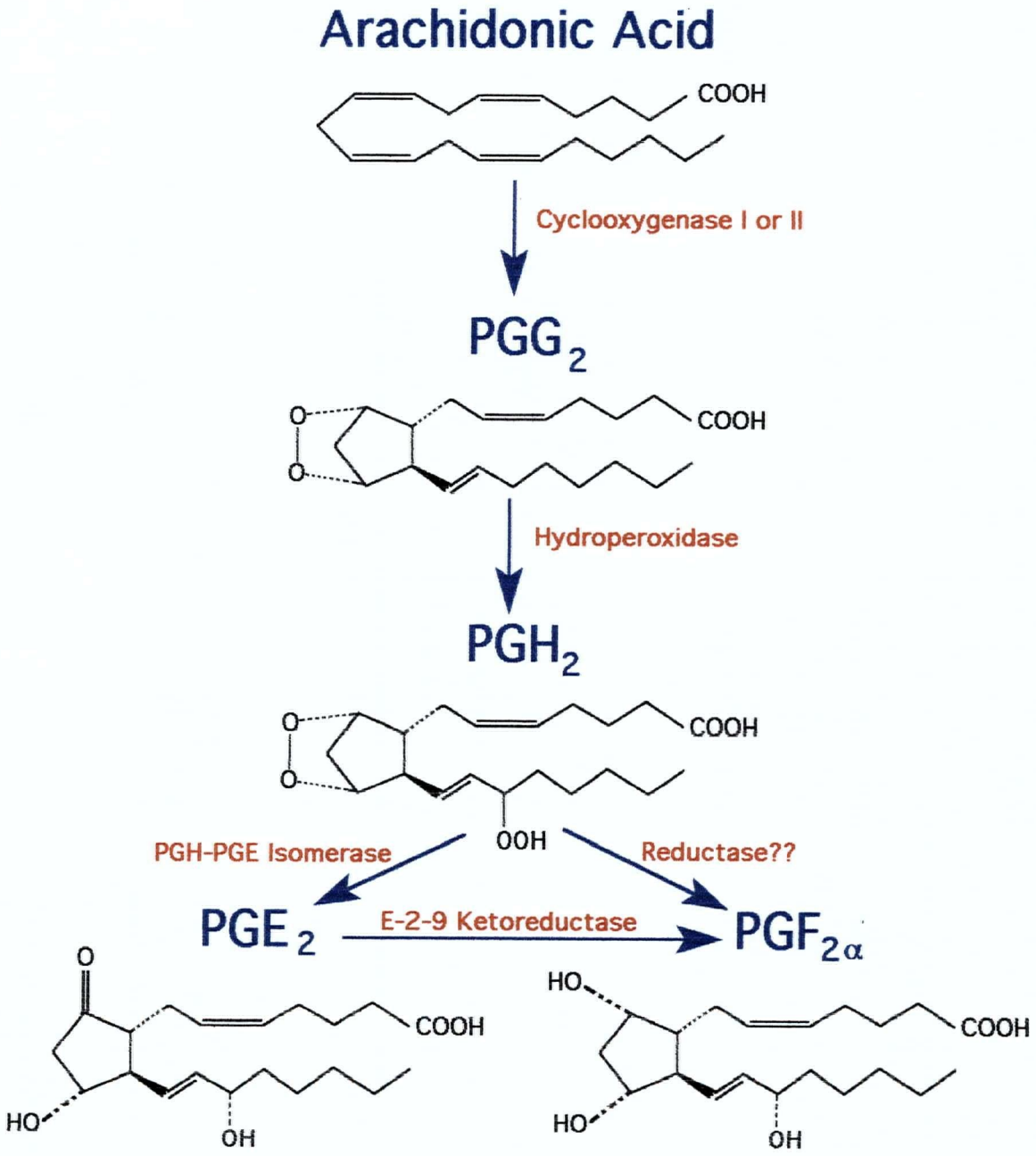


Figure 7. Synthesis of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from arachidonic acid. Enzymes are in red. Arachidonic acid may be converted to prostaglandin G_2 (PGG_2) by cyclooxygenase I (constitutive) or cyclooxygenase II (inducible). Hydroperoxidase converts PGG_2 into PGH_2. Prostaglandin E_2 is produced from PGH_2 by PGH-PGE isomerase. The enzyme E-2-9 ketoreductase converts PGE_2 to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by hydroxylation of the ketone group of PGE_2. Theoretically, a reductase could produce $PGF_{2\alpha}$ by reducing PGH_2, although this pathway has never been demonstrated.

Prostanoid Receptors

Numerous prostanoid receptors have been cloned from mammalian tissues. These receptors include the PGD_2 receptor (DP), the PGE_2 receptors (EP_1, EP_2 and EP_3-family), the $PGF_{2\alpha}$ receptor (FP) and the prostacyclin or PGI_2 receptor (IP) [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993]. Based on sequence analysis, these receptors all appear to belong to the seven-transmembrane G-protein coupled receptor family.

The DP, IP and EP_3-family of receptors are all coupled to cAMP regulation [Adam et al., 1994; Boie et al., 1994 and 1995; An et al., 1994], while the DP, FP, EP_1 and EP_3-family of receptors are coupled to rises in intracellular calcium [Abramovitz et al., 1994b; Adam et al., 1994; Boie et al., 1995; Funk et al., 1993; An et al., 1994]. Additionally, the human EP_3-family of receptors is capable of inhibiting cAMP production through a pertussis toxin-sensitive G-protein [An et al., 1994].

Prostaglandins as Autocrine/Paracrine Factors

Prostaglandins are believed to be autocrine or paracrine hormones. There are numerous lines of evidence pointing to the local nature of prostaglandin actions, these include the following [Smith et al., 1985]:

1) Prostaglandins have a short half life (minutes) *in vivo*, which probably prevents them from having effects systemically. This short half life is mainly due to local degradation by prostaglandin dehydrogenase(s), and systemic degradation by the lung.

2) Prostaglandins are secreted in short (1-5 min) bursts, likely preventing systemic hormone levels from becoming elevated.

3) Most cells that secrete prostaglandins also possess receptors for these hormones, suggesting that they are acting locally.

4) Almost every tissue produces prostaglandins and prostanoid receptors, although these prostanoids produce radically different actions from one tissue to another.

Inhibition and Degradation of Prostaglandins

Cyclooxygenase I and II are short lived enzymes as they are capable of undergoing self-catalyzed destruction. Thus they have been dubbed a suicide enzymes [Smith and Borgeat 1985]. This self catalyzed destruction acts as a negative feedback mechanism on prostaglandin synthesis.

As mentioned above, prostaglandins have very short half lives. The rapid degradation of prostaglandins is due to molecular instability, local degradation by tissue specific hydoxyprostaglandin dehydrogenases and systemic degradation in the lung and kidney [Smith and Borgeat 1985]. It has been reported that circulating PGE_1, PGE_2 and $PGF_{2\alpha}$ are degraded on their first pass through the lung. The lung acts as a filter, by removing virtually all active prostaglandins from the circulatory system. Degradation is achieved by removal of the hydroxyl group at carbon 15 by a NADPH-dependent 15-OH prostaglandin dehydrogenase. Removal of this hydroxyl group reduces the biological activity to ten percent of its original level. Prostaglandin D_2 and PGI_2 are dehydroxylated by another 15-OH dehydrogenase which is specific to these prostaglandins. This second enzyme is found in the kidney.

Further degradation of these prostaglandins occurs via the reduction of the Δ^{13} double bond by an NADPH-dependent Δ^{13} reductase, resulting in 15-keto-13, dihydroprostaglandins which are biologically inactive. Oxidation in the liver and excretion in the urine complete the process.

E. Prostaglandin $F_{2\alpha}$ in Reproduction

Localization of Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ has been detected in the human decidua, amnion, pregnant myometrium and ovary [Satoh et al., 1981; Aksel et al., 1977]. In the human ovary, $PGF_{2\alpha}$ has been localized to the follicle and theca-, granulosa- and luteal-cells [Aksel et al., 1977; Patwardhan and Lanthier, 1981; Plunkett et al., 1975]. Further, the presence of $PGF_{2\alpha}$ has been detected in the human follicle at all stages of the reproductive cycle [Patwardhan and Lanthier, 1981]. Additionally, $PGF_{2\alpha}$ synthesis has been detected in human luteal and stromal tissues where arachidonic acid derived PGE_2 is converted to $PGF_{2\alpha}$, via E-2-9-ketoreductase [Watson et al.,1979; Endo et al., 1988].

Regulation of $PGF_{2\alpha}$ Production in the Ovary

In the ovary, $PGF_{2\alpha}$ production is regulated by a number of ovarian hormones including luteinizing hormone, human chorionic gonadotrophin (hCG), interleukin-1, and tumor necrosis factor [Patwardhan and Lanthier, 1981; Plunkett et al., 1975; Mitsuhashi, 1981; Watanabe et al., 1993; Zolti et al., 1990]. In the rabbit, oxytocin has been suggested as another secretagogue [Fuchs, 1988]. Thus far, propranolol and norepinephrine are known to be receptor-mediated inhibitors of $PGF_{2\alpha}$ production in the human ovary [Bennegard et al., 1984]. However, hCG or cAMP pretreatment has been shown to inhibit the antigonadotrophic actions of cloprostenol ($PGF_{2\alpha}$ analogue) in the luteal cell [Michael and Webley, 1991b].

Functions of $PGF_{2\alpha}$

Prostaglandin $F_{2\alpha}$ has been shown to mediate functional luteolysis and luteal regression, in the mammalian ovary [Michael and Webley, 1991b; Jalkanen et al., 1987; Korda et al., 1975; Grinwich et al., 1976; Moon et al., 1986; Hanzen, 1984; Richardson and Masson, 1980]. However, the presence of $PGF_{2\alpha}$ in the ovary only roughly correlates with this action, as $PGF_{2\alpha}$ levels are highest in mid- rather than late luteal-phase in the human. This discrepancy has been accounted for with the examination of PGE_2, which is known to counteract $PGF_{2\alpha}$ induced luteolysis. Prostaglandin E_2 levels in mid-luteal phase are high while they are not in late-luteal

phase. Thus it is postulated that during the mid-luteal phase, the ratio of $PGF_{2\alpha}$:PGE_2 is low and not suitable for luteolysis, although in the late-luteal phase this ratio is high allowing for luteolysis in the human [Pathwardhan and Lanthier, 1985]. Prostaglandin $F_{2\alpha}$ is known to inhibit LH-, hCG- and PGE_2-stimulated progesterone production (functional luteolysis). Potential mechanisms for functional luteolysis include the inhibition of LH/hCG receptor levels and/or binding [Luborsky et al., 1984], a reduction in adenylate cyclase activation [Dorflinger et al., 1984], increased progesterone catabolism through 20-alpha-hydroxysteroid dehydrogenase [Moon et al., 1986] and possibly an increase in cAMP phosphodiesterase activity via PKC [Lahav et al., 1989; Michael and Webley, 1991a]. Luteal regression is believed to be effected through a $PGF_{2\alpha}$-mediated reduction in blood flow to the corpus luteum and apoptotic cell resorption [Hanzen, 1984; Khan et al., 1989; Richardson and Masson 1980; Quirk et al., 1995].

The luteotrophic action of $PGF_{2\alpha}$ appears to be time-, concentration- and species-dependent. These actions are reported to be strongest in the mid-luteal phase and during pregnancy of investigated species [Khan et al., 1989; Michael and Webley, 1993; Webley et al., 1989; Suginami et al., 1976]. Moreover, ***in vitro*** and ***in vivo*** studies have demonstrated the luteotrophic effects of $PGF_{2\alpha}$ in the presence of gonadotrophin [Suginami et al., 1976], suggesting that the mere presence of gonadotrophins is not sufficient to initiate a luteolytic response from $PGF_{2\alpha}$.

$PGF_{2\alpha}$ in Pregnancy

Studies have demonstrated that temporal and confounding relationships of ovarian hormones may be important in preventing CL regression, should pregnancy occur [Michael and Webley, 1991b]. For example, $PGF_{2\alpha}$ is well accepted as being able to inhibit hCG-stimulated progesterone production in studies where these two hormones are administered together. However, when hCG treatment preceeds $PGF_{2\alpha}$, this luteolytic effect is not seen [Michael and Webley, 1991b]. Similarily, prolactin, LH and FSH, alone and in combination, were not capable of blocking $PGF_{2\alpha}$-induced luteolysis. However, pretreatment with prolactin, FSH plus LH prevented $PGF_{2\alpha}$-induced luteolysis in 11/14 hamsters [Harris and Murphy, 1981]. The blockade of luteolysis by pretreatment with hCG is suggested as being a means by which the placenta rescues the corpus luteum (CL) from $PGF_{2\alpha}$-mediated regression [Webley et al., 1991], thus allowing pregnancy to proceed.

Prostanoid Receptors in Reproductive Tissues

Prostaglandin $F_{2\alpha}$ lowers both gonadotrophin- and prostaglandin E_2-stimulated rises in cAMP, as well as increases intracellular calcium and inositol phosphates in reproductive tissues [Davis et al., 1989; Currie et al., 1992; Pepperell et al., 1989; Lahav et al., 1987]. It is unknown if the actions of $PGF_{2\alpha}$ are exerted through a single or multiple–receptors. Prostaglandin $F_{2\alpha}$ and PGE_2 are both present and active in the human granulosa and luteal cells [Grinwich et al., 1976; Richardson and Masson, 1980; Pathwardhan and Lanthier, 1985; Satoh et al., 1981; Watson et al., 1979]. Thus, it is probable that multiple prostanoid receptors exist in these cells. Furthermore, the currently cloned prostanoid receptors all possess varying degrees of cross–reactivity with PGE_2 and $PGF_{2\alpha}$ [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993; An et al., 1994].

Ligand binding studies have demonstrated that the human $PGF_{2\alpha}$-receptor binds $PGF_{2\alpha}$ with an equilibrium dissociation constant (Kd) of approximately 1 to 1.63 nM [Abramovitz et al., 1994; Lake et al., 1994]. The binding characteristics of the rat $PGF_{2\alpha}$-R suggest a two site model, with a high affinity site (Kd = 3.9 nM) and a lower affinity site (Kd = 34 nM) [Lake et al., 1994].

$PGF_{2\alpha}$ Signal Transduction

Prostaglandin $F_{2\alpha}$-receptor cDNA sequences appear to suggest a G-protein coupled receptor [Lake et al., 1994; Abramovitz et al., 1994], as with other cloned prostanoid receptors [Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993], although pharmacological studies toward this end have not been done in the human ovary. Immunocytochemical studies have localized four different G-protein alpha subunits to the human granulosa-luteal cell including $G_{\alpha S}$, $G_{\alpha i3}$, $G_{\alpha i1,2}$ and $G_{\alpha p}$ (namely $G_{\alpha q}$ and $G_{\alpha 11}$), but not $G_{\alpha O}$ [Lopez et al., 1995]. Furthermore, it has been demonstrated in these cells that cAMP production is regulated by the ratio of $G_{\alpha S}$ and $G_{\alpha i}$, while rises in inositol phosphates and intracellular calcium appear to be regulated by $G_{\alpha p}$ (namely $G_{\alpha q}$ and $G_{\alpha 11}$) and $G_{\alpha i}$ [Lopez et al., 1995].

Exposure of mammalian granulosa or luteal cells to $PGF_{2\alpha}$ has been shown to stimulate phospolipase-C and its downstream pathways [Dorflinger et al., 1984; Abayasekara et al., 1993; Davis et al., 1989; Currie et al., 1992; Michael et al., 1993]. It has been suggested that $PGF_{2\alpha}$ is inhibiting cAMP- and progesterone-production via this rise in inositol phosphates and/or calcium [Leung, 1985; Steele and Leung, 1993]. A direct link between these two pathways has not been clearly established, as numerous reports have demonstrated $PGF_{2\alpha}$-mediated luteolysis in the presence of inositol phosphate, calcium and calmodulin inhibitors [Jalkanen, 1987; Michael and Webley, 1993; Pepperell et al., 1989; Lahav et al., 1987]. Moreover, $PGF_{2\alpha}$ and GnRH stimulate phospholipase-C (PLC) in young, and mid but not in old corpora lutea, suggesting that inositol phospholipid metabolism by itself is not sufficient to explain the luteolytic effects of these hormones [Lahav et al., 1988; Endo et al., 1992]. Further confusing the issue, there are reports of PLC products stimulating progesterone production. Luteinizing hormone can stimulate [Davis et al., 1989; Richards et al., 1995], and has been shown to even potentiate $PGF_{2\alpha}$-stimulated IP_3 production [Davis et al., 1989]. Thus, the possibility of these messengers being responsible for the luteotrophic effects of $PGF_{2\alpha}$ also exists.

Prostaglandin-$F_{2\alpha}$ is known to increase PKC [Abayasekara et al., 1993a,b] and intracellular calcium levels [Currie et al., 1992]. Additionally, PKC activators have been shown to reduce hCG-stimulated cAMP levels. These results suggest that PGF_{2a} exerts its inhibition of hCG-stimulated cAMP and progesterone production via PKC [Abayasekara et al., 1993a,b]. Furthermore, it is believed that inhibition of hCG-stimulated cAMP levels may occur at the level of G_s, as cholera toxin stimulated progesterone production is blocked by $PGF_{2\alpha}$.

Clinical Applications of $PGF_{2\alpha}$

In the female, $PGF_{2\alpha}$ has been utilized for contraception and the induction of abortion or parturition [Concannon and Hansel 1977; Lau et al., 1980; Cameron and Baird 1988; Baird et al., 1988]. Conversely, cyclooxygenase inhibitors such as indomethacin have been used effectively to arrest premature labour and delivery [Manaugh and Novoy 1976; Fuchs et al., 1976].

Prostaglandin $F_{2\alpha}$ is capable of contraceptive effects in the human as well as in some other mammals [Singh and Dominic, 1986; Bilinska and Wojtusiak, 1988; Orlicky and Williams, 1992; Chinoy et al., 1980]. Investigation has revealed the presence of $PGF_{2\alpha}$-receptors on the

Leydig cell, although not on cells of the tunica albuginea, subcapsular- or peritubular-stroma, peritubular boundary tissue, vasculature, spermatogonia, spermatocytes, spermatids, spermatozoa or Sertoli cells [Orlicky and Williams, 1992].

In the mouse, suppressed spermatogenesis and a significant reduction in the weights of the testis, epididymis and accessory sex glands have been reported following $PGF_{2\alpha}$ administration [Singh and Dominic, 1986]. Moreover, seminiferous tubules were found to be devoid of spermatazoa, while Leydig cells showed atrophy. Interestingly, these regressive changes were reversible, as 56 days after drug withdrawl a normal state was achieved [Singh and Dominic, 1986]. Prostaglandin $F_{2\alpha}$ treated rats exhibited reduced testicular- and epididymal-weight, while the weight of their seminal vesicle and ventral prostate increased. Additionally, altered morphology and reduced density- and motility-spermatazoa were seen [Chinoy et al., 1980].

Aside from morphological changes, Leydig cell-androgen production has been reported to be reduced by a $PGF_{2\alpha}$-mediated inhibition of delta 5,3 beta-hydroxysteroid dehydrogenase activity [Bilinska and Wojtusiak, 1988].

F. Gonadotrophin-Releasing Hormone

GnRH Functions

GnRH- and GnRH-receptor mRNA have recently been isolated in the human granulosa cell, indicating that GnRH probably has important local actions within the ovary [Peng et al., 1994]. GnRH is a decapeptide that was first discovered in the hypothalmo-pituitary axis. As mentioned above GnRH is the primary mediator of gonadotrophin release. Gonadotrophin-releasing hormone has also been shown to have luteolytic as well as luteotrophic effects [Leung 1985] in some mammals. Buserelin (a GnRH agonist) has been reported to block hCG, PGE_2 epinephrine and cholera-toxin stimulated progesterone production, as well as potentiating $PGF_{2\alpha}$-inhibition of cAMP production [Massicotte, 1984]. On the contrary, GnRH administration has been utilized to maintain pregnancy or enhance fertility in the cow [Farin and Estill 1993; Funston and Seidel 1995].

GnRH Localization

In humans and other mammals, at least two molecular forms of GnRH have been demonstrated in the brain, ovary and other tissues [King et al., 1990; Ireland et al., 1988; Aten et al., 1987; Behrman et al., 1989; King et al., 1989]. The amount of GnRH in luteal tissues is reported as being proportional to the weight of these tissues, although the concentration of GnRH peptides drops as the corpus luteum develops. While GnRH and/or GnRH peptides are found in numerous nonovarian tissues, in cattle they appear to be relatively concentrated in granulosa cells [Ireland et al., 1988] and pituitary.

GnRH Receptor

Gonadotrophin-releasing hormone is capable of reducing progesterone production and interrupting reproductive cycles and pregnancy in the rat [Clayton et al., 1979]. These actions have been attributed to specific high-affinity receptors present in luteal cell membranes [Clayton et al., 1979; Latouche et al., 1989]. Additionally, this action appears to be autocrine in nature as both GnRH- and GnRH receptor (GnRH-R)-mRNA have been detected within the human

granulosa-luteal cell [Peng et al., 1994]. Moreover, GnRH is reported to autoregulate its own mRNA level as well as those of GnRH-R. Conversely, hCG has been shown to down-regulate GnRH receptor mRNA levels. Messenger RNA for GnRH has also been cloned from the rat corpus luteum, where it was found to have an identical sequence to the rat anterior pituitary GnRH receptor [Whitelaw et al., 1995]. Furthermore, the expression of GnRH-R gene in granulosa cells is purported to be individually regulated for each follicle, to persist in the corpus luteum and is expressed in atretic follicles [Whitelaw et al., 1995; Minaretzis et al., 1995]. In fact atretic follicles appear to exhibit the greatest degree of GnRH-R gene expression, suggesting that GnRH is important in the induction of follicular atresia [Bauer and Jameson, 1995].

GnRH Signal Transduction

In the pituitary gonadotroph, GnRH is known to stimulate polyphosphoinositide breakdown [Kiesel et al., 1986]. On the other hand, both GnRH and NaF-stimulated LH release can occur in the absence of inositol phosphate production [Hawes et al., 1992], suggesting that inositol triphosphate is not an essential second messenger for the release of LH. The question remains which second messengers are necessary for the release of LH from the gonadotroph. Phosphatidic acid, a phospholipase D product, has been reported to increase dose- and time-dependently (2-3 fold; 1-2 min) following GnRH analogue administration in alpha T3-1 cells [Netiv et al., 1991].

Pituitary adenylate cyclase activating polypeptide (PACAP)-stimulated cAMP production is inhibited by GnRH in the alpha T3-1 gonadotroph cell line, although GnRH did not inhibit PACAP binding to gonadotrophs nor forskolin- or cholera toxin-stimulated cAMP production. Thus it has been suggested that the inhibitory effects are exerted at early stages in the signal transduction pathway distal to receptor occupancy but preceeding cAMP production [McArdle et al., 1994], possibly at the level of a G-protein.

Gonadotrophin-releasing hormone and $PGF_{2\alpha}$ both inhibit cAMP production in the corpus luteum. Phosphatidyl inositol (PI) and phosphatidic acid (PA) turnover occurs rapidly (2 and 5 min respectively) with a mean effective dose of 15 and 100 nM for GnRH and $PGF_{2\alpha}$, respectively [Leung, 1985; Davis et al., 1984; Davis et al., 1986]. When co-treatment with the hormones is performed, their effects appear to be additive. Incidentally, A23187 (a pore-forming calcium ionophore) also causes a dramatic increase in PA and PI turnover. Dibutryl-cAMP and

8-Br-cAMP attenuate GnRH and $PGF_{2\alpha}$ stimulated PA and PI turnover. The biproducts of PLC activity (IP3 and DAG) mobilize intracellular calcium, activate PKC and release arachidonic acid [Davis et al., 1986; Shinohara et al., 1985]. The similarity of GnRH and $PGF_{2\alpha}$ responses has led to the suggestion that they may share post-receptor signalling mechansisms [Leung, 1985].

GnRH Mechanism of Action

Studies in the rat have demonstrated GnRH-mediated inhibition of progesterone production through increased activity of 20-alpha-hydroxysteroid dehydrogenase, inhibition of pregnenolone production and reduced activity of P450SCC and 3-beta-hydroxysteroid dehydrogenase activity [Jones et al, 1983; Srivastava et al., 1994]. The mechanisms by which GnRH exerts its luteotrophic effects are not reported in the literature.

Clinical Applications of GnRH

Potent and long-lasting GnRH analogues (super-active agonists) originally developed with fertility promotion in mind have, in fact, proven to have anti-fertility properties in the male and female [Molcho et al., 1984; Bhasin et al, 1984; Nillius, 1985]. These compounds have been applied to numerous therapeutic applications in the female including contraception, treatment of central precocious puberty, and sex steroid-dependent benign and malignant diseases of the reproductive organs [Nillius, 1985]. One of the most common uses of GnRH agonists is the down-regulation of pituitary function in preparation for IVF treatment. Inhibition of ovulation by continuous GnRH agonist administration appears to be safe, reliable and reversible in women [Nillius, 1985]. However, attempts to inhibit luteal function, induce luteolysis or early abortion have not been very successful [Nillius, 1985].

In the human male, high dose GnRH administration interrupts testicular function leading to azoospermia. However, the incompleteness of this azoospermia and unacceptable side effects (loss of libido and potency) rule out the use of GnRH as a male contraceptive [Nillius, 1985]. There have, however, been reports of reduced side effects with co-administration of testosterone [Bhasin et al, 1984; Nillius, 1985].

Interestingly, GnRH is also capable of improving rather than impairing fertility in some species such as the bovine [Farin and Estill 1993; Funston and Seidel 1995].

II – HYPOTHESIS

Prostaglandin $F_{2\alpha}$ is a multi-functional hormone capable of luteolytic and luteotrophic effects in the human granulosa-luteal cell. Moreover, these effects are time-, concentration- and confounding factor-dependent.

III – SPECIFIC OBJECTIVES

A. To define the steroidogenic response of human GLCs to $PGF_{2\alpha}$ with respect to the effects of time in culture, hormone concentration and follicle-size.

B. To examine the potential interactions of $PGF_{2\alpha}$ and GnRH with respect to steroidogenesis.

C. To examine the potential interactions of $PGF_{2\alpha}$ and PGE_2 with respect to steroidogenesis.

D. To define the signal transduction pathways involved in $PGF_{2\alpha}$-mediated luteolysis. Additionally, to define the signal transduction pathway(s) or mechanism(s) by which $PGF_{2\alpha}$ exerts its luteotrophic actions.

E. To examine the regulation of $PGF_{2\alpha}$-R mRNA levels by $PGF_{2\alpha}$.

For a diagramatic depiction of the specific objectives which these studies sought to satisfy please refer to Figure 8. For the rationale (p. 3), results (p. 54), discussion (p. 117) and a synopsis of the findings (p. 143) for each of these objectives refer to the corresponding character (i.e. A, B, C, D & E) in the respective section.

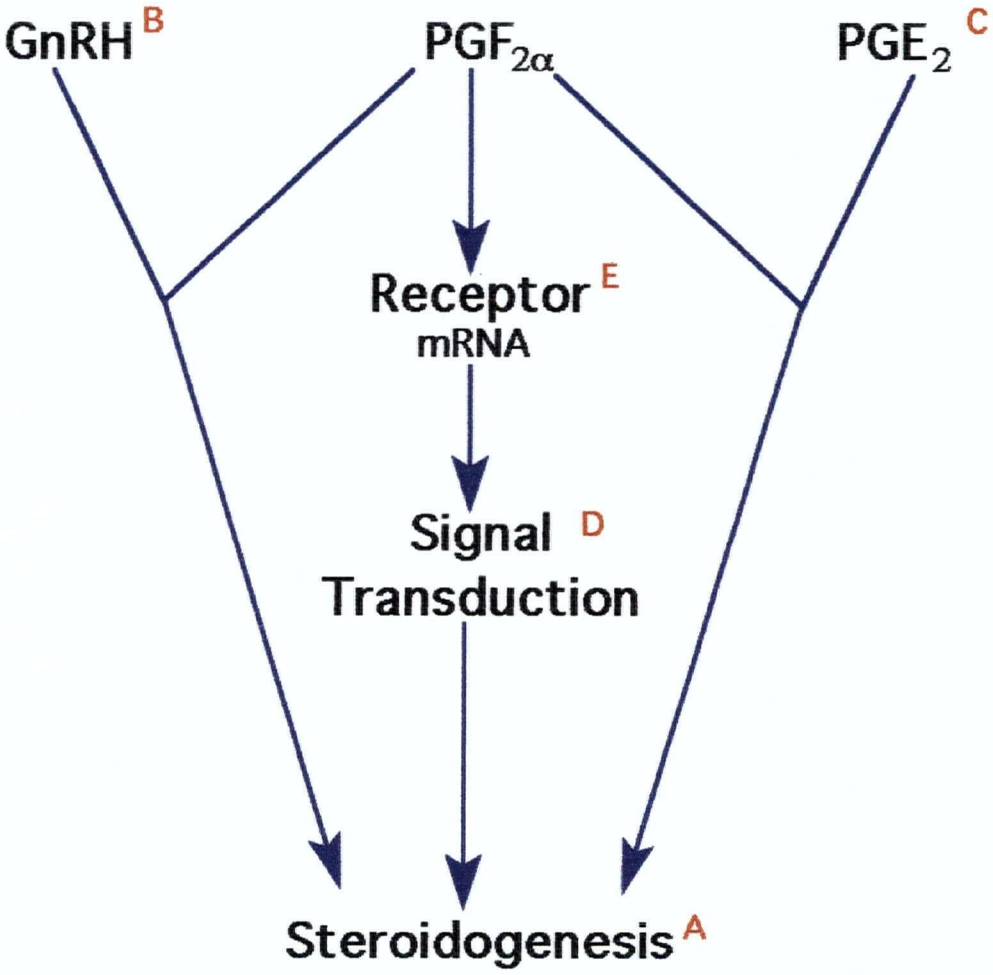


Figure 8. A diagramatic depiction of the specific objectives to be satisfied in these studies. Note that the characters A, B, C, D and E refer to the specific objectives presented above (p. 1). These studies sought to examine the following: A) the effects of $PGF_{2\alpha}$ on progesterone and estradiol production; B) the potential interactions of GnRH and $PGF_{2\alpha}$ on steroidogenesis; C) the potential interactions of PGE_2 and $PGF_{2\alpha}$ on steroidogenesis; D) the signal transduction pathways involved in $PGF_{2\alpha}$-mediated luteolysis; and E) the effects of $PGF_{2\alpha}$ on $PGF_{2\alpha}$-receptor mRNA levels. For the rationale (p. 3), results (p. 54), discussion (p. 117) and a synopsis of the findings (p. 143) for each of these objectives please refer to the corresponding character (i.e. A, B, C, D & E) in the respective section.

IV - RATIONALE

Progesterone and estradiol are key hormones in the regulation of all aspects of the reproductive cycle and pregnancy (as reviewed above, p. 12). Thus the examination of the regulation of these two hormones by $PGF_{2\alpha}$ should reveal, in a very real sense, its effects on reproduction as a whole. If $PGF_{2\alpha}$ were to regulate either of these two hormones in any significant fashion, this would suggest that this hormone is a very important regulator of the human female reproductive system.

Reports on the effects of $PGF_{2\alpha}$ on estradiol production are scant to non-existent. Thus the underlying studies report estradiol in addition to progesterone responses wherever possible (i.e. sample volume permitting).

The rationale for each group of studies corresponding to the specific objectives follows.

A. The Effects of $PGF_{2\alpha}$ on Steroidogenesis

Prostaglandin $F_{2\alpha}$-receptors have been demonstrated in and have been recently cloned from human ovarian cells. These findings suggest that $PGF_{2\alpha}$ may play an important role in the regulation of ovarian function. However, very few functional studies have been performed in the human granulosa cell. Thus the role of $PGF_{2\alpha}$ remains unclear. The conditions under which the luteotrophic and luteolytic functions of $PGF_{2\alpha}$ exist have not been adequately defined. Furthermore, the majority of previous reports examined the effects of $PGF_{2\alpha}$ in the μM range of concentrations, while the reported equilibrium dissociation constants (Kd) of cloned prostanoid receptors fall within the nM range [Abramovitz et al., 1994; Lake et al., 1994]. Therefore, these studies utilized $PGF_{2\alpha}$ at concentrations ranging from 1 pM to 1 μM in order to provide a more complete understanding of the nature of estradiol and progesterone responses to $PGF_{2\alpha}$. There exists the potential that $PGF_{2\alpha}$ is not only important in corpus luteum regression, but also that its temporal relationship to hCG may play a role in the maintenance of early pregnancy. Not only is an understanding of $PGF_{2\alpha}$ important for basic science, but it could also be important clinically.

B. $PGF_{2\alpha}$ and GnRH Interaction Studies

Historically, GnRH has been considered a modulator of gonadotrophin secretion from the gonadotroph. As such, GnRH analogs have been used extensively in both experimental and clinical settings for the modulation of the hypothalamopituitary axis in various situations including: IVF [Pellicer et al., 1992; Gonen et al., 1991; Segars et al., 1990], contraception [Fraser, 1993] and control of amenorrhoea [Martin et al., 1990]. It is only recently that GnRH has been identified in the human ovary, and suggested as a potential local regulator of human ovarian function [Oikawa et al., 1990; Peng et al., 1994]. In order to understand any unwanted *side-effects* of GnRH use in these applications, it is important to further elucidate the local actions of GnRH in the ovary and human granulosa cell.

Gonadotrophin-releasing hormone is believed to share common functions (both luteolytic and luteotrophic actions) and signal transduction pathways (IP_3 and PKC) with $PGF_{2\alpha}$. As the focus of these studies has been to examine the effects of $PGF_{2\alpha}$ in the human ovary, GnRH has been examined primarily in its relationship to potential interactions with $PGF_{2\alpha}$.

C. $PGF_{2\alpha}$ and PGE_2 Interaction Studies

As described above, in the human granulosa-luteal cell $PGF_{2\alpha}$ and PGE_2 exert opposing actions on cAMP-levels and progesterone-production [Grinwich et al., 1976; Richardson and Masson, 1980; Pathwardhan and Lanthier, 1985; Satoh et al., 1981; Watson et al., 1979]. Prostaglandin $F_{2\alpha}$ and PGE_2 can decrease or increase cAMP-levels and progesterone-production, respectively. Prostaglandin $F_{2\alpha}$ is reported to be at its highest concentration during the mid-luteal phase, although it is reported to be luteolytic during the late-luteal phase. The temporal discrepancy between these two events is accounted for by the levels of PGE_2 during these two phases. It has been suggested that high levels of PGE_2 during the mid-luteal phase may prevent premature corpus luteum regression. However, this explanation fails to account for the fact that $PGF_{2\alpha}$-levels are (perhaps 'unnecessarily') at their highest during the mid-luteal phase when conception and implantation occur. A more comprehensive explanation for the elevated levels of $PGF_{2\alpha}$ during the mid-luteal phase may be necessary. Thus, these studies examined the interactions of $PGF_{2\alpha}$ and PGE_2 with respect to steroidogenesis in human GLC *in vitro*.

D. Signal Transduction Studies

In order to fully understand the actions of a hormone, it is essential to know the mode of these actions. Therefore, these studies undertook to examine the signal transduction pathways involved in $PGF_{2\alpha}$-mediated luteolysis and luteotrophism. The post-receptor events involved in the luteotrophic and luteolytic actions of $PGF_{2\alpha}$ are at present speculative.

As the $PGF_{2\alpha}$-receptor [Lake et al., 1994; Abramovitz et al., 1994] is known to belong to the seven transmembrane G-protein coupled receptor family, studies focused on the potential role of G-proteins in the mediation of luteolysis and luteotrophism. Prostaglandin $F_{2\alpha}$ has been shown to lower gonadotrophin- and PGE_2-stimulated progesterone production (through a lowering of cAMP levels), and G-proteins are known to regulate cAMP levels within these cells. This study examined the role of G-proteins in mediating the effects of $PGF_{2\alpha}$. Pertussis-toxin (PTX) and cholera-toxin (CTX) were utilized to elucidate the potential role of G-proteins in the anti-gonadotrophic actions of $PGF_{2\alpha}$. In order to determine the action(s) of $PGF_{2\alpha}$ distal to G-proteins in the signal transduction cascade, these studies examined the ability of $PGF_{2\alpha}$ to inhibit progesterone production induced by activators of the adenylate-cyclase, and by cyclic adenosine monophosphate (cAMP) analogues.

Previous studies have demonstrated a correlation between the effects of $PGF_{2\alpha}$ and a rise in inositol phosphate metabolism [Leung, 1985; Steele and Leung, 1993]. Moreover, a number of studies have demonstrated altered responses to $PGF_{2\alpha}$ in the presence of PKC modulators. However, there is much controversy in the literature over the importance of inositol phosphates and PKC in the luteolytic effects of $PGF_{2\alpha}$ [Jalkanen, 1987; Michael and Webley, 1993; Pepperell et al., 1989; Lahav et al., 1987]. The underlying studies sought to confirm or disaffirm the existence of of PKC-mediated alteration in the luteolytic effects of $PGF_{2\alpha}$, although an exhaustive examination of this pathway was not performed. An explanation for the apparent discrepancies in the literature is proposed based on these studies and the known pathways by which prostaglandins are known to act in other systems.

E. $PGF_{2\alpha}$-R mRNA Studies

Prostaglandin $F_{2\alpha}$ is known to act though receptor mediated mechanisms. Thus the regulation of $PGF_{2\alpha}$-R levels is as important as the regulation of $PGF_{2\alpha}$ itself. Receptor binding studies have previously demonstrated the presence of $PGF_{2\alpha}$-R in the rat and bovine luteal cell [Brambaifa et al., 1984; Bussmann et al., 1989]. Moreover, the existence of $PGF_{2\alpha}$-R mRNA has recently been demonstrated in the human granulosa-luteal cell [Ristimaki et al., 1997]. However, there have been no reports on the regulation of $PGF_{2\alpha}$-R mRNA levels in response to $PGF_{2\alpha}$. Thus these studies examined the ability of $PGF_{2\alpha}$ to regulate $PGF_{2\alpha}$-R mRNA levels.

V – MATERIALS AND METHODS

A. Granulosa-Luteal Cell Collection and Culture

The use of human GLC was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British Columbia. Granulosa-luteal cells were harvested in conjunction with oocyte collection in the University of British Columbia's *in vitro* fertilization program. Throughout the pre-collection period, follicular development was monitored using estradiol assays and ultrasonography. After pituitary down-regulation with a GnRH analogue (Synarel, Syntex; Montreal, PQ) and when estradiol levels were less than 150 pmol/l, follicular development was stimulated with hMG (Humegon 75 IU FSH and 75 IU LH, Organon, Scarborough, ON; or Fertinorm 75 IU FSH, Serono, Oakville, ON). When three or more follicles reached a diameter greater than 16-18 mm, and estradiol levels were greater than 5000 pmole/l, final maturation was induced with hCG (10,000 IU; Serono). Thirty-two to thirty-six hours later oocytes were harvested using a transvaginal approach. Granulosa-luteal cells were harvested from the follicular fluid following oocyte identification and removal.

Following centrifugation (1,000 x g) of the follicular contents, the supernatant was decanted and cells were resuspended in medium 199 (M199; Gibco-BRL Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Gibco). This step was repeated to provide a second wash. Following the second wash, the resuspended cells were layered on top of a mixture of Percoll (40%; Sigma, St. Louis, MO) diluted in M199. This gradient was centrifuged (1,700 x g), for 10 min at 22 C. Following collection from the M199/Percoll interphase, granulosa cells were washed and resuspended (10^5-10^6 cells/0.5 ml) in M199, supplemented with 10% FBS, sodium penicillin (100 IU/ml; Gibco) and streptomycin (100 μg/ml; Gibco), and plated on 48-well plates (Corning, NY; 0.5 ml cell suspension/well).

Cells to be used in one-day (D_1) pre-cultured experiments were cultured for 24 h and then used. However, cells to be used in eight-day (D_8) and twelve to fourteen-day ($D_{12\text{-}14}$) pre-culture experiments had media changed every 2-3 days until the cells had been cultured for a total of 8 or 12–14 days, respectively. A pre-incubation (wash; 1 h) with fresh M199 was performed prior to experimental incubations in order to rinse the cells. All incubations were at 37 C, in a humidified, water-jacketed incubator (5% CO_2; Forma Scientific Inc, Mississauga, ON). The methods utilized in these studies are depicted in Figure 9.

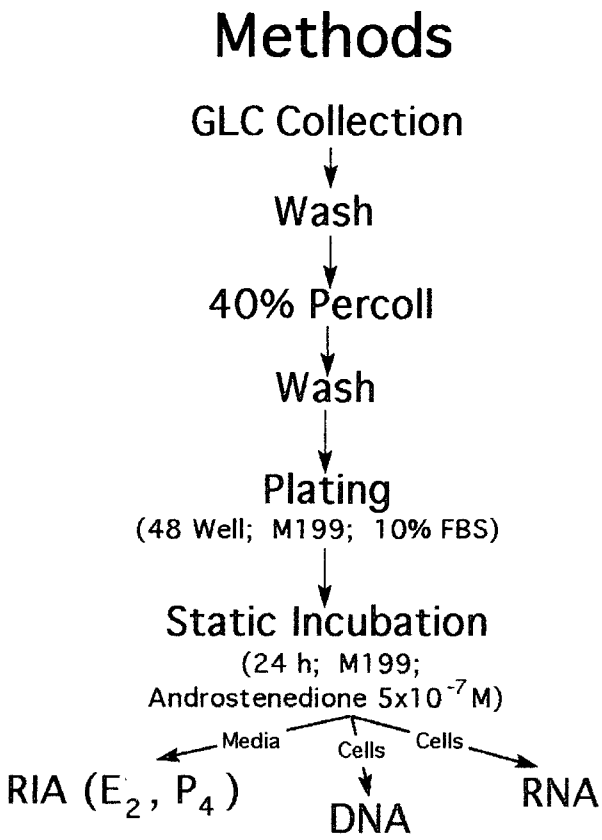


Figure 9. Schematic of methods utilized in studies presented herewithin. In short, human granulosa luteal cells (GLC) are collected during oocyte collection from patients undergoing *in vitro* fertilization. Cells were washed twice and then separated from red blood cells on a 40% Percoll density gradient (in Medium 199), after which cells were washed twice and plated at 10^3 to 10^4 cells/well (on a 48 well tissue culture plate), in medium supplemented with 10 % foetal bovine serum (FBS). After culturing for one, eight or twelve to fourteen days of culture cells were preincubated (washed) for 1 h in fresh medium and then subjected to hormonal or pharmacological treatments in medium (24 h), supplemented with substrate for estradiol production (androstenedione 5 x 10^{-7} M). Supernatant was then collected and stored (-20 C) until assayed for progesterone and estradiol. Cells were either extracted for DNA or total RNA which were assayed with a Hoechst dye DNA assay or reverse-transcription/semi-quantitative polymerase chain reaction, respectively.

B. Incubation Experiments

All treatment regimens were performed in serum free Medium-199 or Dulbecco's Minimum Essential Medium) supplemented with androstenedione (5×10^{-7} M; precursor for estradiol formation). Following a 24 hour treatment, media were removed and stored at -20 C until assayed for progesterone or estradiol concentrations. The hormones and pharmacological agents utilized in these studies are presented in Table 2. The concentrations of these agents utilized were selected based on their known pharmacology.

The duration of these release experiments posses potential problems with receptor down-regulation or desensitization, however, this treatment duration was chosen to increase the probability of attaining measurable steroid levels in the release media.

Viability was checked post-experiment by the ability of cells to exclude trypan blue. Viability as approximated by this method was greater than 95% at all culture-time periods and under all treatment regimens.

The following experiments were performed:

1. Human Chorionic Gonadotrophin Concentration Response Curve: D_8 cultured GLCs were treated with vehicle or hCG (0.001 to 10 IU/ml).

2. Culture Time- and Concentration-Dependent Responses to $PGF_{2\alpha}$ and GnRH: Day_1, D_8 and $D_{12\text{-}14}$ cultured human GLCs were treated with vehicle, hCG (1 IU/ml) or hCG plus $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M). A similar experiment was performed with GnRH in place of $PGF_{2\alpha}$.

3. Follicle Size Dependent Changes in hCG and $PGF_{2\alpha}$ Responses: Cells were also separated based on follicle size (> and < 12 mm in diameter) and subjected treatment with vehicle, hCG (1 IU/ml), $PGF_{2\alpha}$ (10^{-6} M), or hCG plus $PGF_{2\alpha}$ (10^{-11} to), at D_1. Ideally, follicles should have been separated into more categories. However due to clinical limitations this was not possible.

Table 2. Hormones and pharmacological agents utilized in these studies.

#	Name	Abbrev.	Class	Target(s)	Concentration(s)
1	Androstenedione	None used	Steroid Hormone Precursor	Estradiol Biosynthetic Pathway	5×10^{-7} M
2	Bisindolylmaleimide	PKCi	Enzyme Antagonist	Protein kinase-C	50 nM*
2	Cholera Toxin	CTX	Bacterial Toxin	G Protein α-subunit Gs	1μg/ml
3	Dibutryl-Cyclic-Adenosine Monophosphate	db-cAMP	Second Messenger Analogue	Protein Kinase A	10^{-5} M
4	Forskolin	For	Enzyme Activator	Adenylate Cyclase	10^{-5} M
5	Gonadotrophin Releasing hormone	GnRH	Peptide Hormone	GnRH Receptor	10^{-10} to 10^{-5}
6	Human Chorionic Gonadotrophin	hCG	Peptide Hormone	LH/hCG Receptor	0.001 to 1 IU/ml
7	Indomethacin	Indo	Enzyme Activator	Cyclooxygenase I; Prostaglandin Dehydrogenase	10^{-6} M
8	Isoproterenol	Iso or IsoP	Catecholamine Hormone Antagonist	ß-adrenergic Receptor	10^{-5} M
9	Pertussis Toxin	PTX	Bacterial Toxin	G Protein α-subunit(s): Gi, Gp	50 ng/ml
10	Prostaglandin E_2	PGE_2	Eicosanoid Hormone	PGE_2 Receptor and other Prostanoid Receptors	10^{-12} to 10^{-6} M
11	Prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$	Eicosanoid Hormone	$PGF_{2\alpha}$ Receptor and other Prostanoid Receptors	10^{-12} to 10^{-6} M

* Toullec et al., 1991; McCarthy 1995.

4. Interaction of $PGF_{2\alpha}$ and GnRH: Day_1 and D_8 GLCs were treated with vehicle, $PGF_{2\alpha}$ (10^{-9} M), GnRH (10^{-6} M) or $PGF_{2\alpha}$ plus GnRH, in the absence or presence of human chorionic gonadotrophin (hCG). In a second experiment (D_1 cells), vehicle, $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M) and GnRH (10^{-10} to 10^{-5} M) concentration-response curves were crossed into a matrix of 49 separate treatments which were assayed for progesterone. Results were plotted in three dimensions with GnRH, $PGF_{2\alpha}$ and progesterone-response each on a axis. Similarily, results were also plotted as a contour map with GnRH and $PGF_{2\alpha}$ each on a separate axis and progesterone response represented by shading and colour. Moreover, 'slices' of the three dimensional matrix were plotted in two dimenstions and analyzed statistically.

5. Interaction of $PGF_{2\alpha}$ and PGE_2: Day_8 GLCs were treated with vehicle, $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M) and PGE_2 (10^{-10} to 10^{-5} M) concentration-response curves which were crossed into a matrix of 49 separate treatments. Media were assayed for progesterone. Results were plotted in three dimensions, with $PGF_{2\alpha}$, PGE_2 and progesterone-response each on a separate axis. Similarily, results were also plotted as a contour map, with $PGF_{2\alpha}$ and PGE_2 each on a separate axis and progesterone response represented by shading and colour. Moreover, 'slices' of the three dimensional matrix were plotted in two dimenstions and analyzed statistically, as above.

6. PTX and CTX Effects on $PGF_{2\alpha}$ Mediated Luteolysis: Day_1 and D_8 cells used for G-protein studies were pre-treated (18 h) with M199 supplemented with vehicle, PTX (50 ng/ml), CTX (1 μg/ml), or PTX plus CTX. Following the pre-treatment period, cells were exposed to M199 containing vehicle, PTX, CTX or PTX plus CTX; plus vehicle, hCG (1 IU/ml), $PGF_{2\alpha}$ (10^{-6} M), or hCG plus $PGF_{2\alpha}$, for 24 h. In another set of experiments cells were treated with M199 containing vehicle, IsoP (10^{-5} M), $PGF_{2\alpha}$ (10^{-6} M), or IsoP plus $PGF_{2\alpha}$. Finally, cells were exposed to M199 containing vehicle or $PGF_{2\alpha}$ (10^{-6} M), plus or minus forskolin (10^{-5} M) or Db-cAMP (10^{-5} M).

7. Forskolin and Db-cAMP: Day_8 cultured human GLCs were treated for 24 hours with vehicle and $PGF_{2\alpha}$ (10^{-6} M) with and without forskolin (10^{-6} M) or dibutryl cAMP (db-cAMP; 10^{-5} M).

8. Progesterone and Estradiol Production per Cell or DNA Level: Plots were made of the basal progesterone- and estradiol-production from human GLC versus total cell numbers plated or DNA levels per well. This experiment was performed to determine if there was any correlation between steroid production and cell numbers or DNA levels.

9. Morphology of Human GLCs with Culture Time: Photographs of human GLCs at day zero, one, eight, twelve and sixteen were taken, in order to present the general morphology of cells at these culture times.

Following studies 2 and 3, it was apparent that culture-time radically altered the responses to hormone treatment. Thus, particular attention was paid to culture-time when deciding which response was to be examined with a particular experiment. For example, a luteotrophic response to $PGF_{2\alpha}$ was absent in D_1 cultured GLCs. Therefore, this time period was particularly appropriate for examining the ability of GnRH to elicit a luteotrophic response to $PGF_{2\alpha}$.

C. Microscopy

Cells were routinely checked following plating, prior to experiments and following experiments for viability (as described above) and general appearance with a Nikon TMS inverted tissue culture microscope. Moreover, photographs of cells at different culture periods were taken with either a Nikon N2000 or Contax 167 MT camera body mounted on this microscope, using Fuji Provia (100 ASA) or Fujichrome Tungsten (400 ASA) film.

Slides were scanned with a Power Macintosh 6100AV (72 MB RAM) using a Nikon Coolscan II and printed on a photoenhanced Macintosh Colour Stylewriter 2500 using photograde paper (at > 720 dpi). Colour synchronization was set to automatic photograde.

D. Radioimmunoassay of Progesterone and Estradiol

The progesterone and estradiol concentrations in culture media were determined by specific RIAs, as previously described [Li et al., 1993; Rodway et al., 1990; Leung & Armstrong, 1979], with the following modification: phosphate buffered saline was replaced by a phosphate buffer containing Na_2HPO_4 (0.04 M) and NaH_2PO_4 (0.04 M) at pH 7.4. Typical standard curves for these progesterone and estradiol assays are presented in Figure 10.

Progesterone-RIA was performed as follows. Briefly, the assay used rabbit progesterone antiserum (P_4-2; Kindly provided by D.T. Armstrong, University of Western Ontario) raised against 4-pregnen-6ß-ol-3,20-dione hemisuccinate:bovine serum albumin conjugate (Steraloids, Wilton, NH). The final antiserum concentration was 50 μg/ml. A standard competition method was employed utilizing progesterone (Sigma) standards, and ^{3}H-progesterone at 10,000 cpm/tube (Amersham, Oakville, ON). The range of the assay standards was from 1 to 128 ng/ml. A 0.04 M phosphate buffer (pH 7.4) was used for diluting samples and controls with a final assay volume of 600 μl/tube. Phosphate buffer with dextran (0.025% w/v) and charcoal (0.25% w/v) was used to separate free progesterone from bound. Free progesterone in the supernatant was diluted in 3.0 ml of scintiverse (Fisher) scintillation cocktail and counted for 60 sec on a Wallac 1217 Rackbeta-counter.

The RIA was sensitive to 1.5 ng/ml, as determined by taking the progesterone concentration two times the standard deviation below the zero-binding value. Samples were assayed in duplicate. Intra- and inter-assay coefficients of variation were less than 11%.

Estradiol-RIA used specific rabbit antiserum (D.T. Armstrong) raised against estratriene-3,17ß-diol-6-carboxymethyl-oxime:BSA conjugate (Steraloids). The final antiserum dilution was 1:200,000 w/v in phosphate buffer. As with the progesterone-RIA, a standard competition method was employed, utilizing estradiol (Sigma) standards and ^{3}H-estradiol (Amersham, Oakville, ON) at 10,000 cpm/tube. The estradiol-RIA was performed as described above for the progesterone-RIA. Furthermore, the range and sensitivity was similar to the progesterone-RIA. Intra- and inter-assay coefficients of variation were less than 10%.

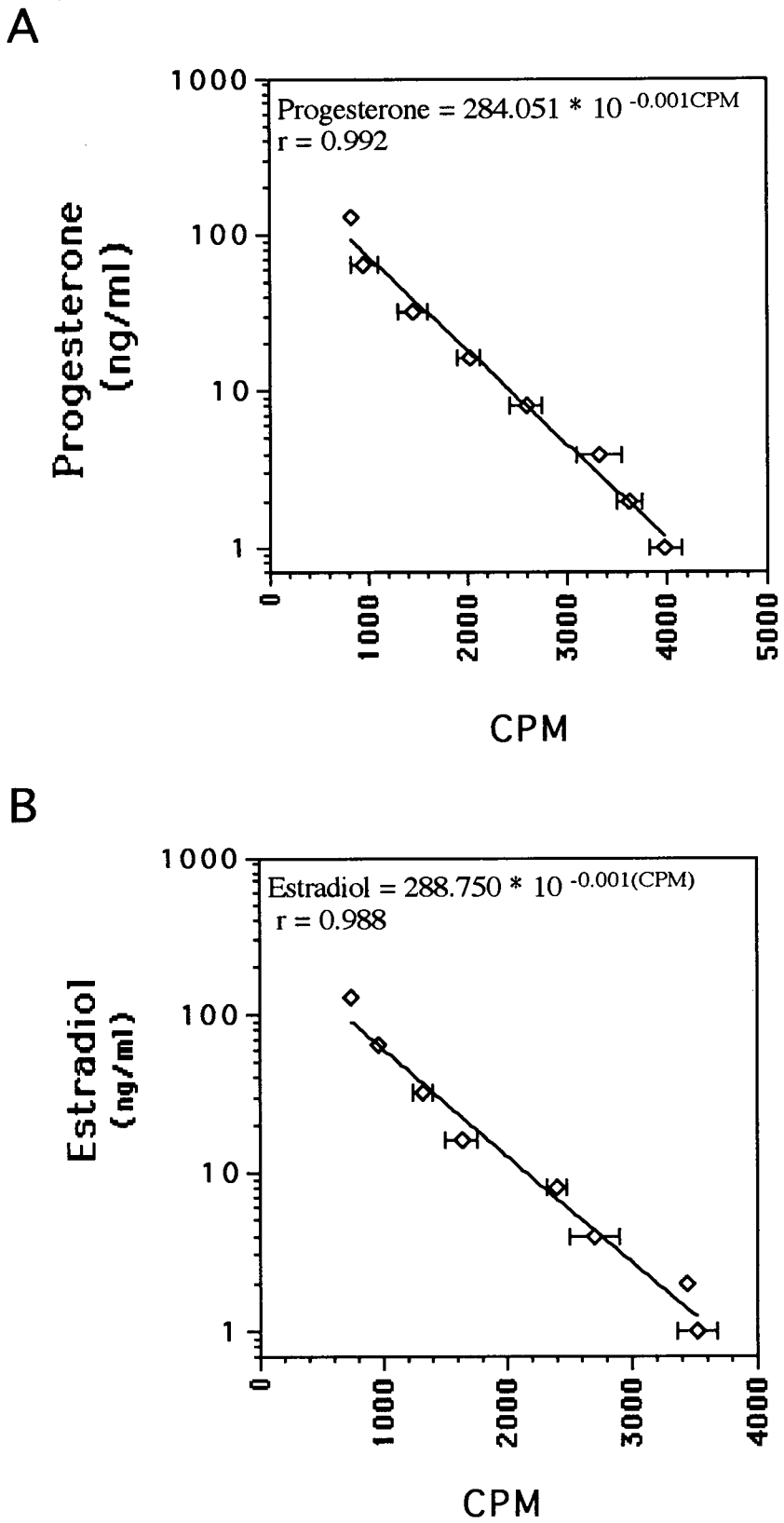


Figure 10. Typical progesterone (**A**) and estradiol (**B**) radioimmunoassay standard curves. Counts per minute (CPM).

E. Hoechst Dye DNA Assay

DNA quantification was performed using a modified version of Mates method [1986]. Briefly, following the treatment period, media were removed and replaced with trypsin TRTPK (50 μg/ml; Sigma) in a final volume of 500 μl in phosphate buffered saline (PBS as defined below). The plate was stored frozen at (-70 C) until assayed for DNA. At the time of assay, the plate was thawed at room temperature and incubated for 30 min to allow the trypsin to lyse the cells. During this incubation period, pre-prepared (see below) Hoechst dye stock (Bisbenzimide; 20 μg/ml in H_2O; Sigma) was thawed (from -20 C) and diluted (10x in PBS). Following the incubation period, Hoechst dye solution was added to each well (at 500 μl/well), mixed and incubated for 5 min before well contents were measured with a spectrofluorometer (Aminco Rowman Spectrophoto Fluorometer, American Instrument Co., Silver Springs, MD) for fluorescence. Excitation and emission wavelengths were 354 and 458 nM, respectively. DNA was quantified by extrapolation from known standards (calf thymus DNA; Sigma) which were prepared by serial dilution (in phosphate buffered saline) over a range of 2.5 to 1000 ng/ml. Standards (1 ml) contained Hoechst dye diluted in similar fashion to samples above. Fluorescence was measured as above, with standards being measured in triplicate.

Hoechst dye stock (20 μg/ml) was slowly dissolved in distilled water, aliquotted (5 ml), wrapped in foil, and then stored at -20 C until use. Foil wrapping was necessary as bisbenzimide is light sensitive and will quench with time.

Phosphate buffered saline (PBS) was composed of Na_2HP0_4 (7.1 g), NaCl (116.88 g), and EDTA (0.84 g), dissolved in 750 ml of water, and then made up to final volume (1.0 l) and pH (7.4). PBS was stored at room temperature until use.

A typical standard curve for this assay is presented in Figure 11.

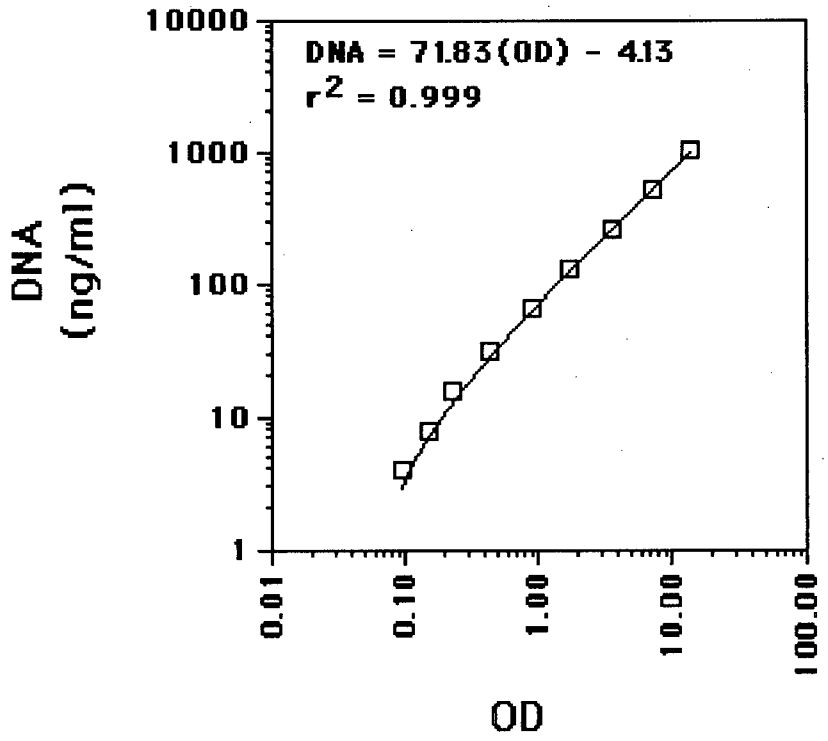


Figure 11. Typical hoechst dye deoxyribonucleic acid (DNA) assay standard curve. Optical density (OD).

F. RNA Extraction Procedure

Following experiments, some plates were stored (at -70C) until extracted for total RNA with an RNaid kit (Bio 101, La Jolla, CA). The extraction procedure was performed as outlined in the kits instructions. Lysis buffer (100 μl, as defined below) was added to each well, mixed with a pipette tip and left on ice for 5 min. The buffer with lysed cells was then transferred to a microcentrifuge tube (1.5 ml; Canlab). Sodium acetate (0.2M; 10 μl; pH 4.0) and phenol (100 μl) were added and vortexed. Chloroform:isoamyl alcohol (24:1; 100 μl) was added, vortexed and the preparation was then left on ice (15 min). Following this incubation, the tubes were spun (10,000 g; 20 min; 4 C) with the top phase being collected afterwards. RNA was present in the top phase, while protein and DNA remained in the lower phases. Thus, care was taken not to remove any of the interphase as this would introduce contamination into the RNA extract. A second extraction with chloroform:isoamyl alcohol (24:1; 100 μl) was performed and spun (2 min) with the top phase again being carefully removed and placed in a new microtube.

Vortexed RNAMatrix (10 μl) was added to each tube, vortexed (30 s) and incubated (5 min; RT) with occasional mixing to allow RNA adsorption. Tubes were then centrifuged (1 min; 10,000 g) to pellet the RNA/RNAMatrix complex. Supernatant was removed and saved for possible readsorption. Tubes with the pellet were briefly re-centrifuged and the remaining supernatant was carefully removed with a small bore pipette tip. Following this, the pellet was resuspended in the provided RNA wash solution (500 μl), spun (1 min; 10,000 g), supernatant was removed, and this step was repeated 1 more time. The microfuge-tubes with the pellets were then placed in the speed-vac micro centrifuge(1 min).

Finally, the pellet was resuspended in DEPC treated water (15-100 μl) and incubated (55 C; 5 min) to elute RNA. A final spin (1 min; 10,000 g) was performed to pellet the RNA Matrix while leaving the RNA in solution which was transferred to a final microfuge tube (0.5 ml). The solution was then subjected to spectrophotometric analysis to quantify total RNA.

Lysis buffer was composed of guanidine thyocyanate (4.0 M), sodium citrate (pH 7.0; 5 mM), sarcosyl (0.5 % w/v) and ß-mercapto-ethanol (0.7% v/v) in diethylpyrocarbonate (DEPC) treated water.

G. RNA Gel

In order to check the relative efficacy of the RNA extraction procedure and the integrity of the RNA, the extraction products were run on an RNA gel. The RNA gel was composed of agarose (1.0%) dissolved in dH_2O (21.6 ml). Additionally, RNA gel-running buffer (GRB-R; 3 ml; as defined below) and formaldehyde (5.34 ml) were added, and the solution was allowed to cool (5 min) before pouring into a gel tray. RNA samples were loaded (1-2 μg in 10 μl) along with GLB-R (3 μl), and the gel was run (100 V; 50 min). Staining of the gel with ethidium bromide revealed two RNA bands (18 and 28 S). The gel was then photographed with polaroid 665 positive/negative film.

GLB-R was composed of glycerol (50%), EDTA (1 mM), bromophenol blue (0.4%), xylene cyanol (0.4%) and ethidium bromide. The GRB (10x) consisted of MOPS (0.2 M), NaOAc (80 mM) and EDTA (10 mM) in dH_2O (total volume 1.0 l).

H. Reverse Transcription of RNA to cDNA

A fixed quantity of total RNA, between 1-3 μg depending on the amount available (following RNA extraction) was made up in DEPC treated water (8 μl), heated (70 C; 10 min) and then spun down (5 min; 10,000 g). DTT (1μl), oligo-dT (1μl) and bulk mixture (5 μl) were added, followed by an incubation (37 C; 1 h; Pharmacia First Strand cDNA Kit, Upsala, Sweden). The preparation was boiled for (10 min), spun down and frozen (-20 C) until use. Total RNA levels were determined by spectrophotometric estimation. The spectrophotometer was validated by repeatedly measuring a known quantity of DNA and calculating the error between measurements (see results, p. 110).

I. Polymerase Chain Reaction (PCR)

Complementary DNA obtained from reverse transcription reactions were amplified by PCR such that relative changes in $PGF_{2\alpha}$-receptor expression could be examined. The procedure was performed as follows. A fixed quantity of complementary DNA (cDNA) between 1 to 5 μl depending on availability for each experiment was mixed with a sense and antisense primer (1 μl of each; Table 3), Master Mix (22 μl; as defined below) and Taq polymerase (0.2 μl) in a microcentrifuge tube (0.5 ml; Canlab). Vegetable oil was then dropped on top of the mixture and the tube was capped. PCR was performed for each gene as specified in Table 4, with the primers described in Table 5.

Master Mix was composed of 10x PCR buffer (1/10 vol) plus deoxynucleotide-triphosphates (dNTPs; 0.179 μmol/ml). Ten times PCR buffer consisted of Tris-HCl (100 mM; pH 8.3), KCl (500 nM), $MgCl_2$ (15 mM) and gelatin (0.1%) in ddH_2O. Radiolabelled PCR contained 4.0 nCi of ^{32}P-dCTP.

J. DNA Gel

Polymerase chain reaction products were run on an agarose gel composed of the following. Agarose (1.0%) was dissolved in a Tris-Borate-EDTA buffer (TBE) by boiling for 2 minutes. When the agarose solution had cooled (5-10 min) it was poured into a gel tray and a comb was inserted until the gel had solidified (approx. 20 min). The gel was then submersed in TBE, and cDNA samples (10 μl, with 5-20 μg DNA) mixed with DNA gel-loading-buffer (GLB-D; 3 μl) were loaded. After loading, a DNA ladder (Gibco BRL) was loaded on the outside lanes of the gel, and the gel was run (120-140 V). The gel was removed, stained with ethidium bromide and photographed with a Polaroid camera under ultraviolet light. PCR products appear as fluorescent bands.

TBE (5x) was composed of TRIS-base (10.8 g), boric acid (5.5 g) and EDTA (0.5 M; pH 8.0) dissolved in dH_2O (final volume 1 l). Furthermore, GLB-D consists of glycerol (50 ml), EDTA (0.5 M; 20 ml), bromophenol blue (0.1 g), xylene cyanol (0.1 g) and H_2O (20 ml).

Table 3. Primer combinations and expected product size following PCR.

Sense	Antisense	Predicted Product Size (bp)
hPGF+	**hPGF-**	802
rPGF+	**rPGF-**	720
Act+	**Act-**	524

bp - base pairs

Table 4. PCR conditions utilized for genes examined.

Gene	Denaturing		Annealing		Polymerization		Cycles	Extension	Cycle Expt.
	Temp	Time	Temp	Time	Temp	Time		Time	Figure
$hPGF_{2\alpha}$-R	96	30	57	30	72	1:30	40	7:00	56A
$rPGF_{2\alpha}$-R	96	30	50	30	72	1:30	40	7:00	None
ß-Actin	96	30	55	30	72	1:30	30	7:00	56B

All temperatures are given in degrees C, while times are in minutes:seconds

Table 5. Oligonucleotide sequences utilized for PCR and Southern blot hybridization.

Gene	+/-	Primer sequence (5' to 3')	Name	MW	Ref
human	+	CTC ATG AAG GCA TAT CAG AG	hPGF+	6127	1
$PGF_{2\alpha}$	-	GTT GCC ATT CGG AGA GCA A	hPGF-	5831	
Receptor	+*	GCT TCT GAT AAA GAA TGG ATC CGC TT	hPGFP+	7955	
Rat	+	CCA TTG CCA TCC TCA TGA AGG	rPGF+	6407	2
$PGF_{2\alpha}$	-	AGC GTC GTC TCA CAG GTC AC	rPGF-	6120	
Receptor	+*	CAG TAC GAT GGC CAT TGA GAG GTG CAT	rPGFP+	8399	
ß-Actin	+	TGA TCC ACA TCT GCT GGA AG	Act+	6117	3
Control	-	GAC CTG ACT GAC TCA CTC AT	Act-	6037	

+ = sense; - = antisense. * - utilized as an internal probe for blots. MW - Molecular weight. 1 - Abramovitz et al., 1994. 2 - Lake et al., 1994. 3 - Ng et al., 1985,

K. Southern Blot Hybridization

Southern blot hybridization allows for the verification of PCR products by hybridizing a probe designed to bind to the internal portion of the predicted PCR product. The procedure used was as follows. An agarose gel containing the expected PCR product was denatured by immersion and agitation (15 min; RT) in a solution containing NaCl (1.5 M) and NaOH (0.5 M). Sodium hydroxide was then neutralized with a solution of NaCl (3 M) and Tris (0.5 M) at pH 8.0. Three washes with a sodium chloride/sodium citrate buffer (SSC; as defined below) followed (5 min each), after which an overnight transfer to a nylon membrane was performed (Fig. 12). The SSC buffer was composed of sodium chloride (26.3 g/l), and sodium citrate (13.2 g/l), in dH_2O (pH 7.0).

Following transfer of the gel to a nylon membrane, the membrane was washed (SSC), dried wrapped in Saran Wrap™, and exposed to UV light (2 min). The membrane then was stored (4 C) until hybridization, which was performed with a radiolabelled oligonucleotide, specific to the inner sequence of the predicted PCR product (Table 5).

Radiolabelling of the oligonucleotide was performed by a kination reaction, as follows. Primer (10 pmol; 1 μl), T_4 kinase buffer (1 μl; 10x), dH_2O (2 μl), γ^{32}P-ATP (5 μl) and T_4 kinase (10 U; 1μl) were mixed and incubated (1 h; 37 C). The probe was then boiled (2 min), and spun (1 min; 10,000 g).

Just prior to hybridization the nylon membrane was removed from the refrigerator and preincubated in a prehybridization solution. The probe was then diluted in a hybridization solution and hybridized (40 C; over night). The following day the membrane was washed repeatedly in SSC (Table 6). The washed membrane was blotted, re-wrapped in Saran Wrap™, and then autoradiographed for 20 min to several days (at -70 C) depending on signal strength.

L. Densitometry of Photographed Gels and Autoradiographs

RNA and DNA gels stained with ethidium bromide (200 μg/100 ml gel; Sigma) could be visualized with UV illumination (Photoprep, Bio/Can Scientific, Mississauga, ON). However, quantification of products required gels to be photographed with a negative film (polaroid, 665). Negatives and autoradiographs from Southern blotting were scanned with a transluminescence video densitometer (Model 620, Bio-Rad Laboratories Inc.). Scanning software was utilized to calculate the relative optical density of each product band. In order to reduce variability, three scans of each film were performed and the means of the three scans were plotted.

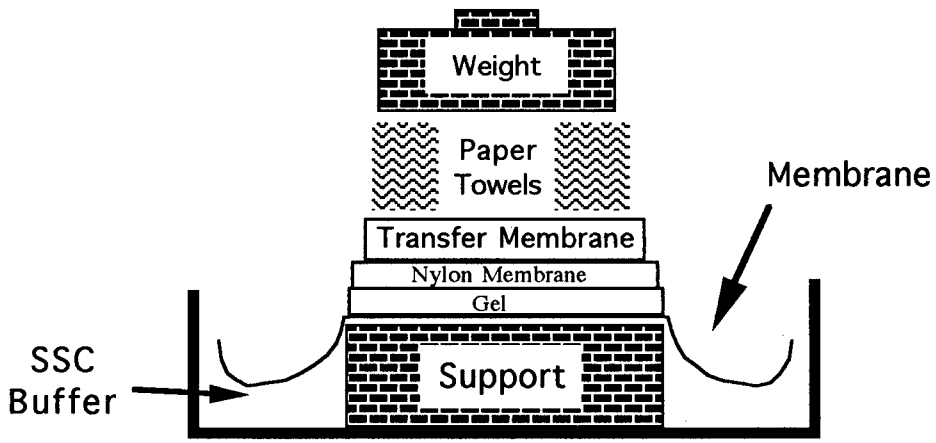


Figure 12. Setup for overnight transfer of gel products to a nylon membrane for further Southern blot hybridization.

Table 6. Southern Blot SSC Washes.

Wash	Duration (Minutes)	Temperature (Celcius)	SSC Dilution
1	20 min	40	1.0x
2	10 min	50	0.1x
3	10 min	50	0.1x

SSC-sodium chloride/sodium citrate buffer.

M. Analysis of Results

The results were presented as percentage of control values or by representative experiment. Graph bars represent the mean ± SEM of experiments performed on cells from different patients ('n' refers to patient numbers). Statistical analysis utilized one-way ANOVA followed by a Fischer or Scheffe post-hoc test. Statistical analysis was performed on mean standard score data and plotted in percentage of control data [Lewis 1984; Fisher and van Belle 1993; Grimm and Yarnold 1996; Porkess 1991]. Different characters above graph bars signify statistical difference.

VI – RESULTS

Preliminary Results

Basal and hCG-Stimulated Steroidogenesis from human GLCs

1. Basal Steroid Secretion per Cell or Level of DNA/Well

No correlation between cell plating number and basal progesterone production was seen in D_8 cultured human GLCs (n=17). Furthermore, up to 5000-fold differences in basal progesterone production from individual patients were observed (Fig. 13A and B). Moreover, when progesterone production was plotted against extracted DNA levels, no correlation was seen between culture-well DNA content and progesterone production (n=19; Fig. 14A and B).

Similar results were seen when basal estradiol production was plotted against cell plating numbers (Fig. 15A and B; n=11) or extracted DNA levels (Fig. 16A and B; n=17). Please see the discussion section for possible explanations for these results (p. 117).

Cell viability as determined by trypan blue dye exclusion was greater than 95% in these experiments, a result further supported by the ability of these cells to respond (with steroid production) to experimental stimuli such as hCG and $PGF_{2\alpha}$ (not shown).

2. hCG-Stimulated Progesterone Production in Cells from 3 Different Patients

Progesterone responses to hCG (0.001 IU/ml) in D_8 precultured human GLCs from three different patients produced significant stimulatory responses ($p<0.05$) of similar magnitude (approximately 4-fold; Fig. 17). However, the basal concentrations of progesterone varied up to 100-fold between experiments performed on cells from different patients, although all three experiments were performed on cells plated at 10^4 cells/well. For example, cells from patient 1 (Fig. 17A) produced basal progesterone levels of approximately 10 ng/ml, while hCG-stimulated progesterone levels were approximately 45 ng/ml. Basal and hCG-stimulated progesterone production were approximately 1 and 4 ng/ml, respectively in cells from patient 2 (Fig. 17B). Finally, basal and hCG-stimulated progesterone production were approximately 110 and 420 ng/ml, respectively in cells from patient 3 (Fig. 17C). Thus, although very different basal levels were seen in all three cases, the relative responses to hCG were similar.

(see p. 117 for relevant discussion)

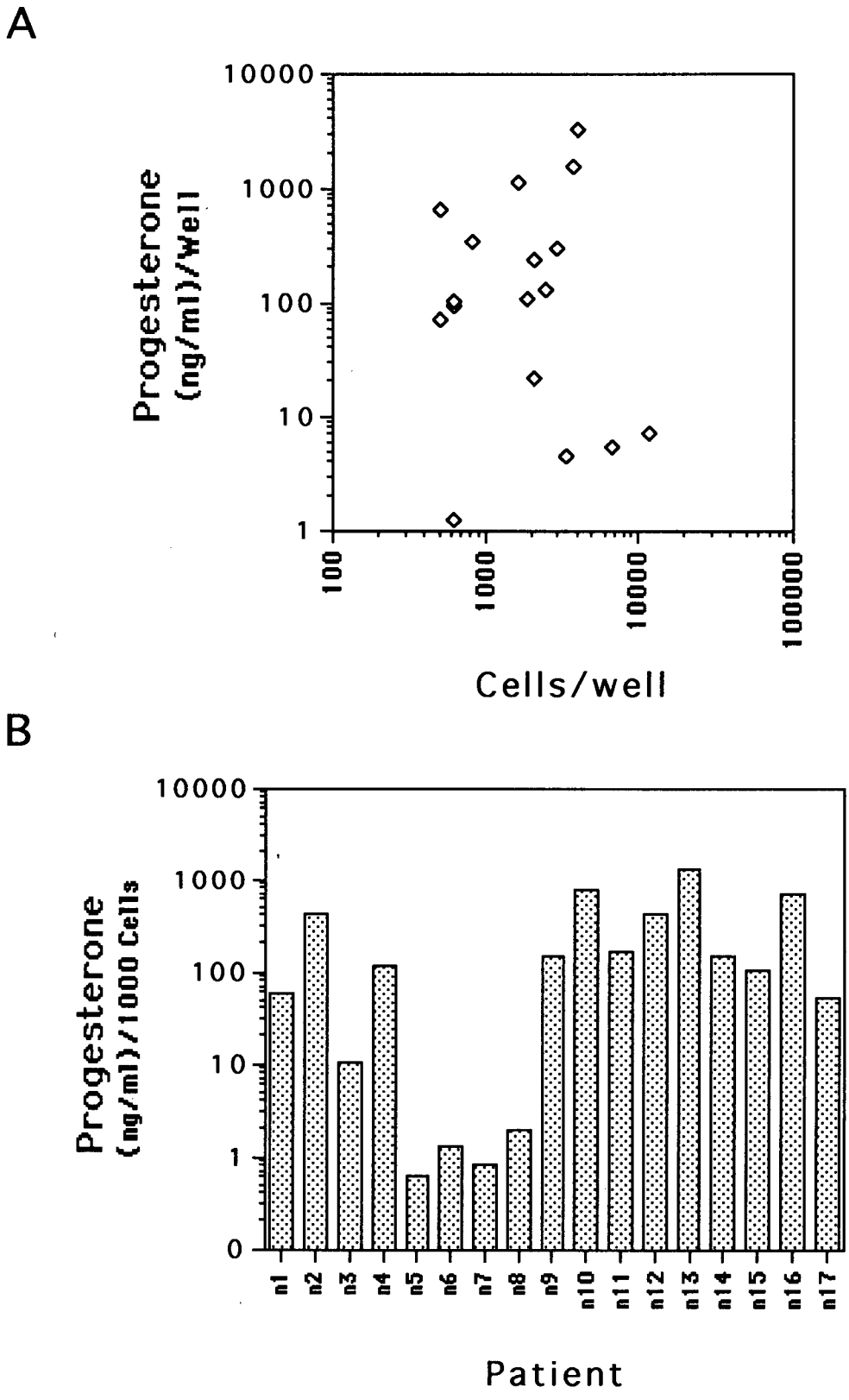


Figure 13. A. Basal progesterone production (ng/ml; over 24 h) versus cells/well, in D_8 pre-cultured human granulosa-luteal cells (GLCs). **B.** Basal progesterone production of individual patients plotted in ng/ml per 1000 cells plated, in D_8 pre-cultured human GLCs. Note that no correlation was seen between plated cell numbers and progesterone production between patients.

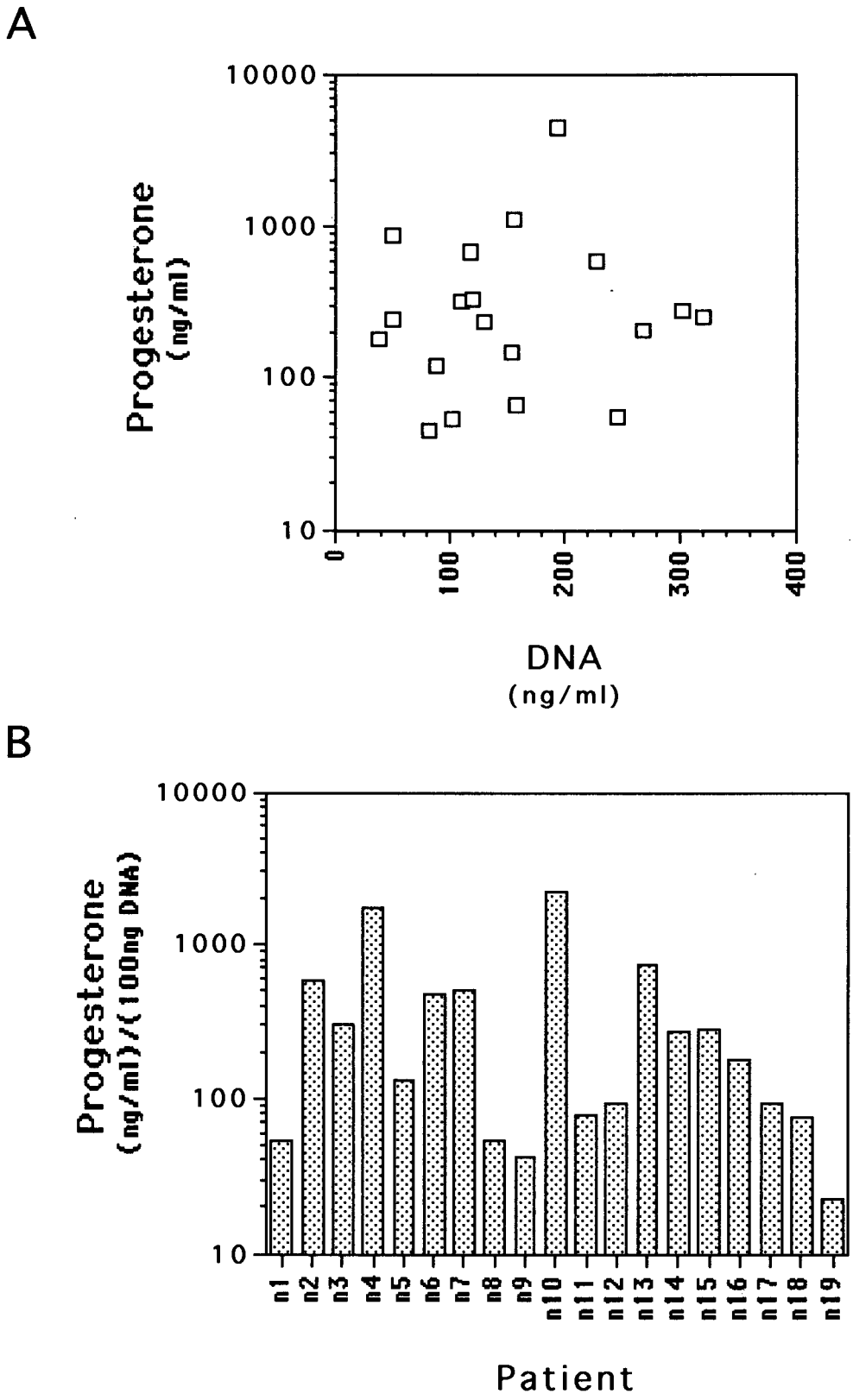


Figure 14. **A.** Basal progesterone production (ng/ml; over 24 h) versus DNA content per well (ng/ml), in D_8 pre-cultured human granulosa-luteal cells (GLCs). **B.** Basal progesterone production of individual patients plotted in ng/ml per 100 ng of DNA, in D_8 pre-cultured human GLCs. Note that no correlation was seen between extracted DNA levels and progesterone production.

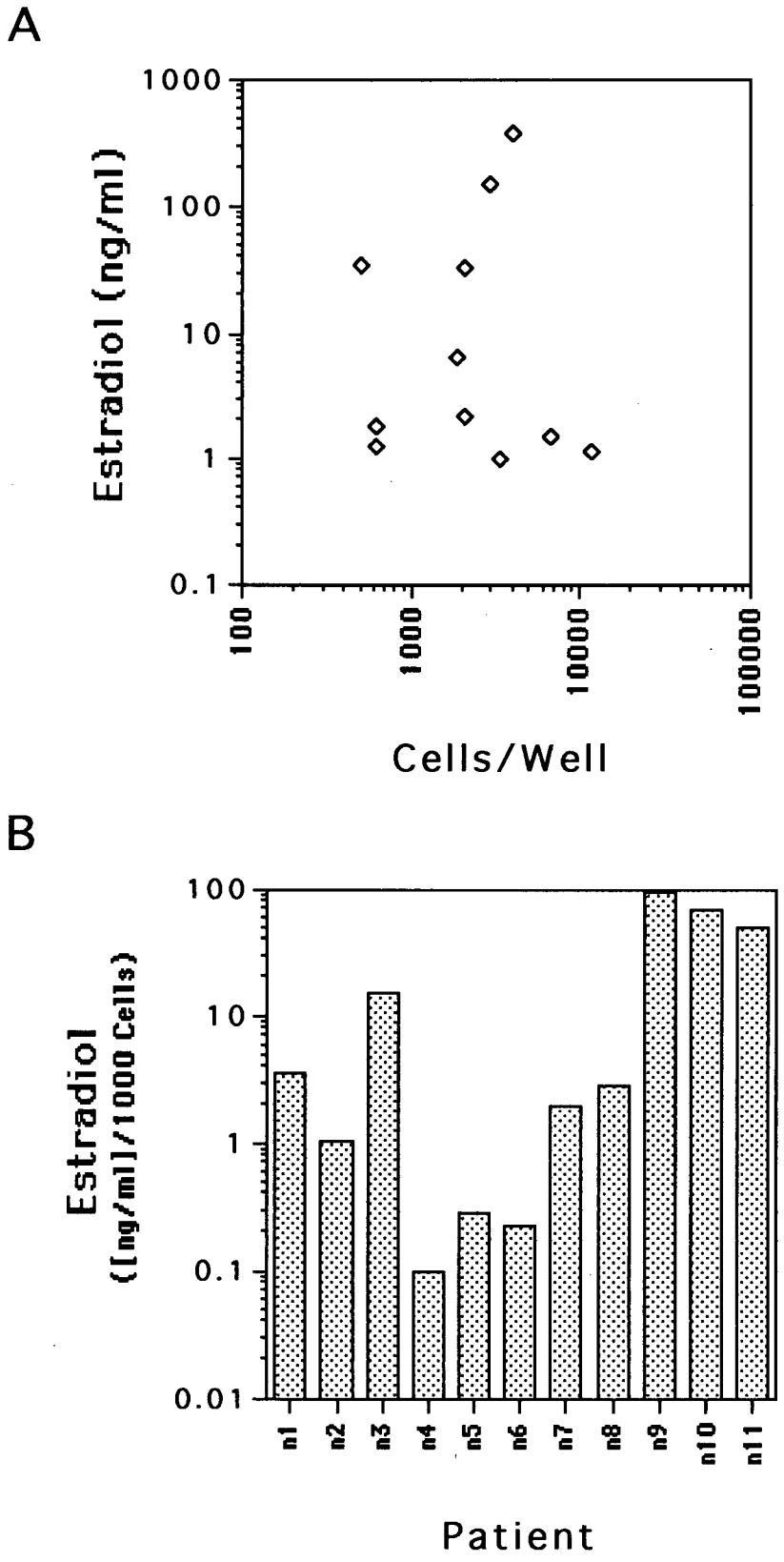


Figure 15. A. Basal estradiol production (ng/ml; over 24 h) versus cells/well, in D_8 pre-cultured human granulosa-luteal cells (GLCs). **B**. Basal estradiol production of individual patients plotted in ng/ml per 1000 cells plated, in D_8 pre-cultured human GLCs. Note that no correlation was seen between plated cell numbers and estradiol production.

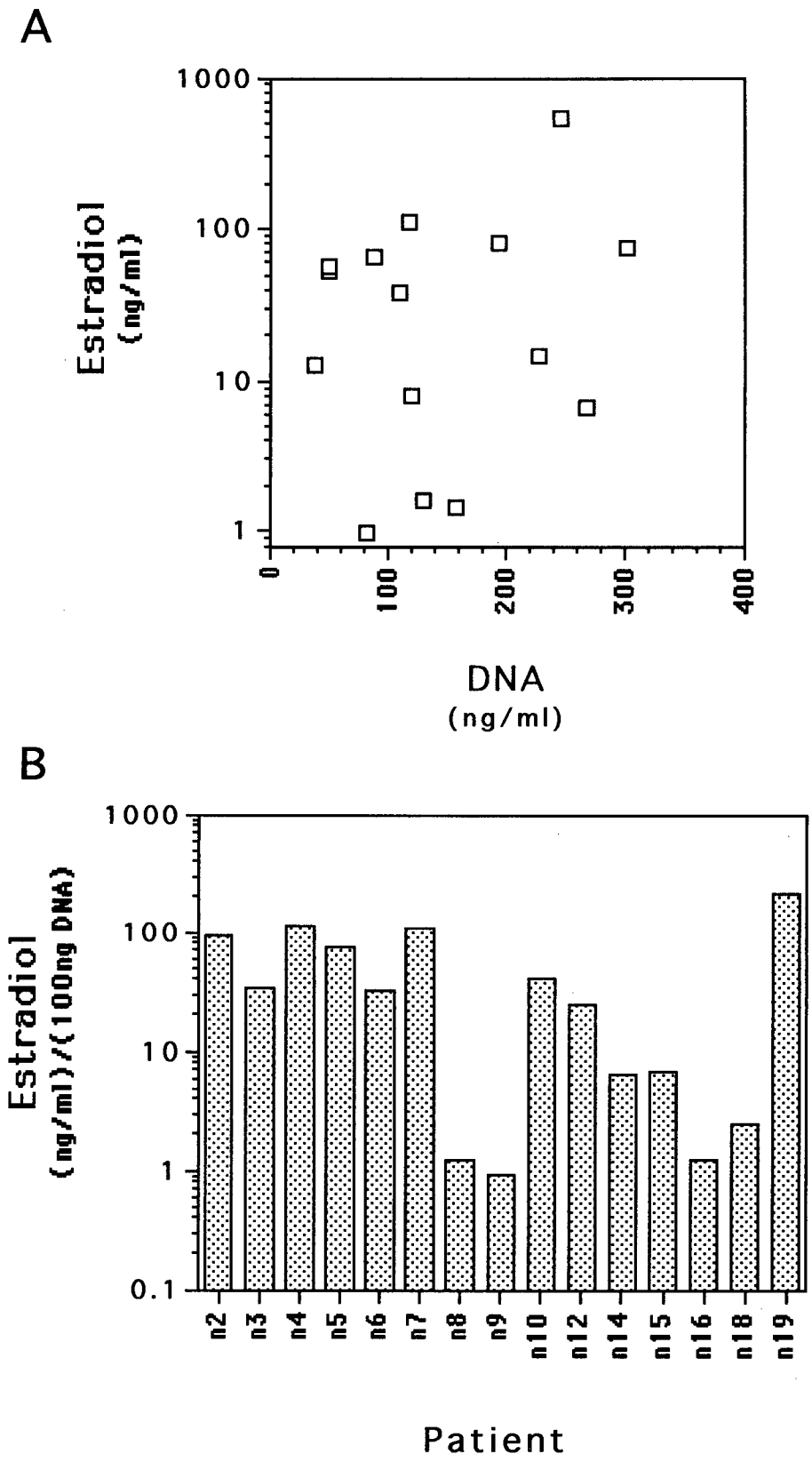


Figure 16. A. Basal estradiol production (ng/ml; over 24 h) versus DNA content per well (in ng/ml), in D_8 pre-cultured human granulosa-luteal cells (GLCs). **B.** Basal estradiol production of individual patients plotted in ng/ml per 100 ng of DNA, in D_8 pre-cultured human GLCs. Note that no correlation was seen between extracted DNA levels and estradiol production.

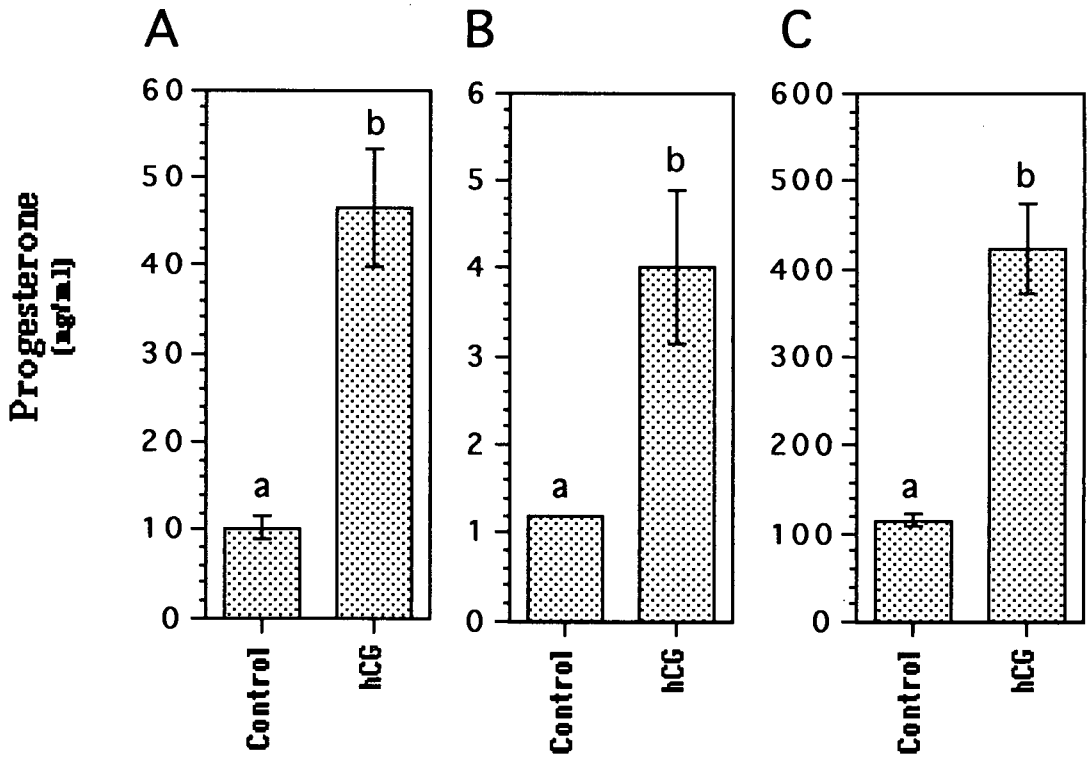


Figure 17. Comparison of progesterone responses to hCG treatment (0.001 IU/ml for 24 h), in D_8 pre-cultured human granulosa-luteal cells from three different patients (**A**, **B** and **C**). Progesterone production was significantly stimulated in all three experiments ($a \neq b$, $p<0.05$). Moreover, all three experiments responded with similar amplitudes (approximately 4 fold). However, the magnitude of progesterone concentrations varied up to 100 fold between experiments performed on cells from different patients. All three experiments were performed on cells plated at 10^4 cells/well.

Human GLC Morphology with Culture Time

Morphology slides presented within this section were taken from cells of a single patient which were plated at 10^4 cells per well and cultured as described above. The morphology of cultured human GLCs plated on 48-well plates changed with culture time. Cells that were freshly plated (2-3 h of culture) appeared to be evenly distributed about the surface of the well (Fig. 18A; 66 x mag). Additionally, cells did not appear to be highly associated with one another, although some cell aggregates were present. Individual cells appeared round and smooth.

Following one day of culture (D_1), GLCs were unevenly distributed throughout the well with clusters of cells being present and empty unpopulated regions throughout the well (Fig. 18B; 66 x mag). Cells were either round and smooth or elongated. Many cells possessed cytoplasmic projections which appeared to form associations with neighbouring cells.

Long term cultures (D_8) of GLC resulted in highly associated cells which were primarily present in clusters with very few cells existing outside of these aggregates (Fig. 19A; 200 x mag). Cells that were not part of a tightly associated aggregate formed contacts with aggregated cells with cytoplasmic projections. Individual cells again appeared round, although not smooth. Cells appeared luteinized, as they were highly irregular and granulated, when compared to early cultures. Even longer term cultures (D_{12}) resulted in cells that were even more associated and irregular in shape than those of D_8 cultures (Fig. 19B; 200 x mag).

Cells cultured for 1 to 12 days remained viable as evidenced by trypan blue dye exclusion (> 95%) and their ability to respond to experimental stimuli with steroid production. However, cells maintained in culture for sixteen or more days were no longer viable or responsive to stimuli and appeared to be luteolysed (Fig. 20; 66 x mag).

Similar culture-time dependent morphological changes were witnessed in all other cultures of human GLCs presented herewithin.

(see p. 120 for relevant discussion)

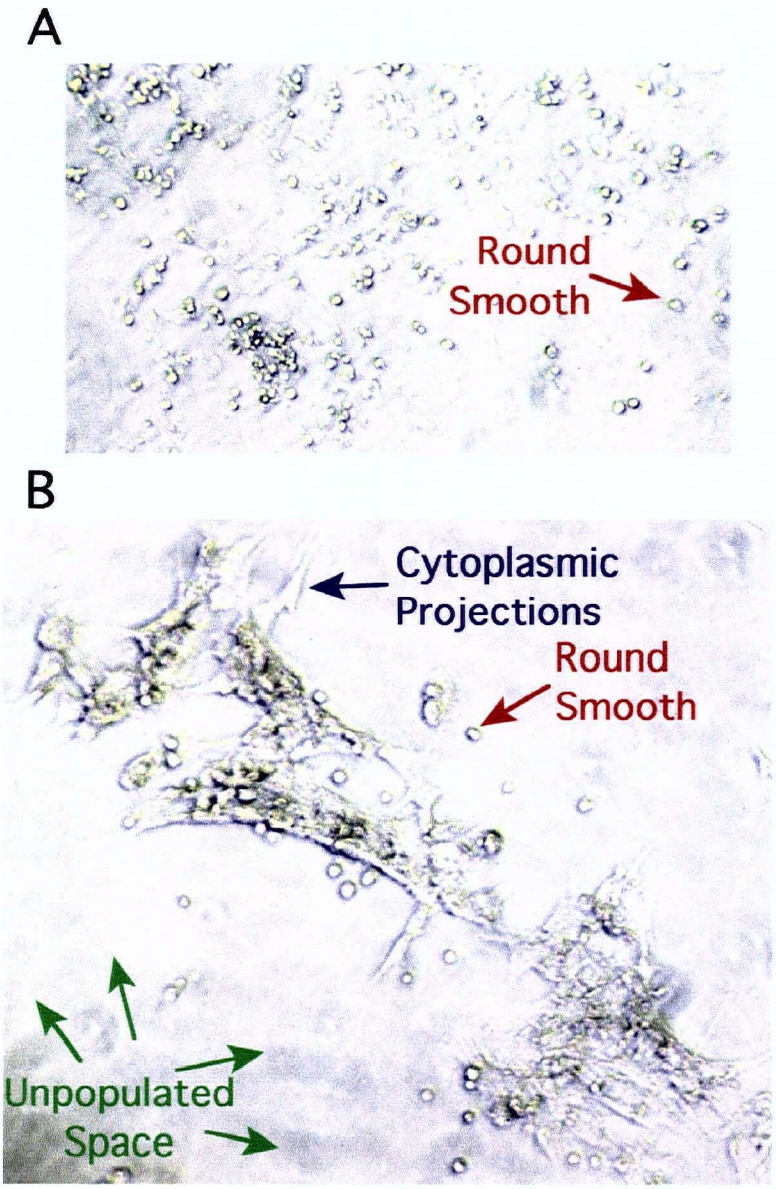


Figure 18. **A.** Human granulosa-luteal cells (GLCs) that were freshly plated (2-3 h of culture) appeared to be evenly distributed throughout the surface of the culture well (40 x mag). Additionally, cells did not appear to be highly associated with one another, although some cell aggregates were present. Individual cells appeared round and smooth. **B.** Following one day of culture GLCs were unevenly distributed, with cell clusters and empty unpopulated regions being distributed throughout the well (66 x mag). Cells appeared to be smooth. Many cells possessed cytoplasmic projections which appeared to form associations with neighbouring cells.

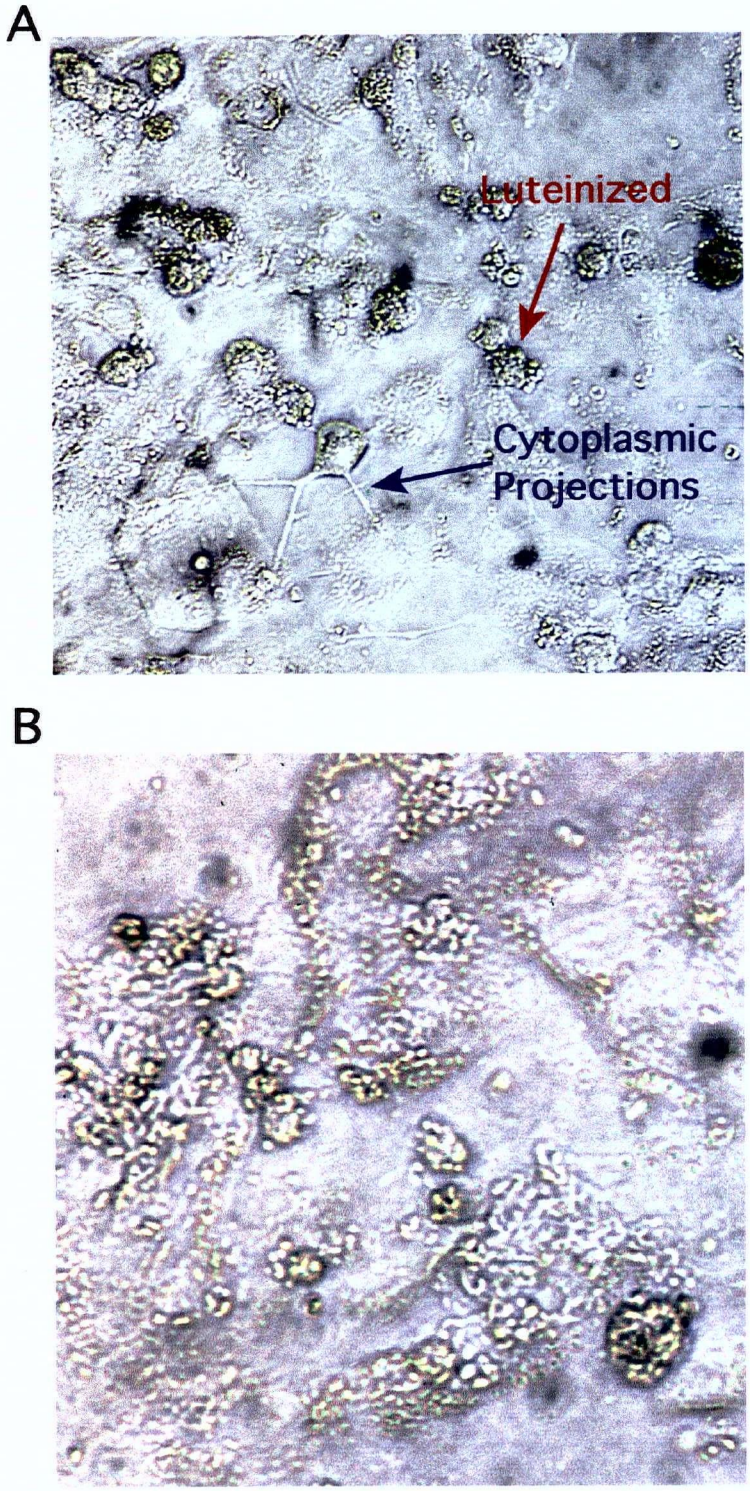


Figure 19. A. Eight day cultures of human granulosa-luteal cells (GLCs) resulted in highly associated cells, which were primarily present in clusters, with very few cells existing outside of these aggregates (200 x mag). Cells that were not part of a tightly associated aggregate formed contacts with aggregated cells with cytoplasmic projections. Individual cells again apppeared round, although not smooth. Cells appeared luteinized, as they appeared blebbed and granulated (or vacuolated), when compared to early cultures. **B.** Twelve day cultured GLCs were highly associated and irregular in shape (200 x mag).

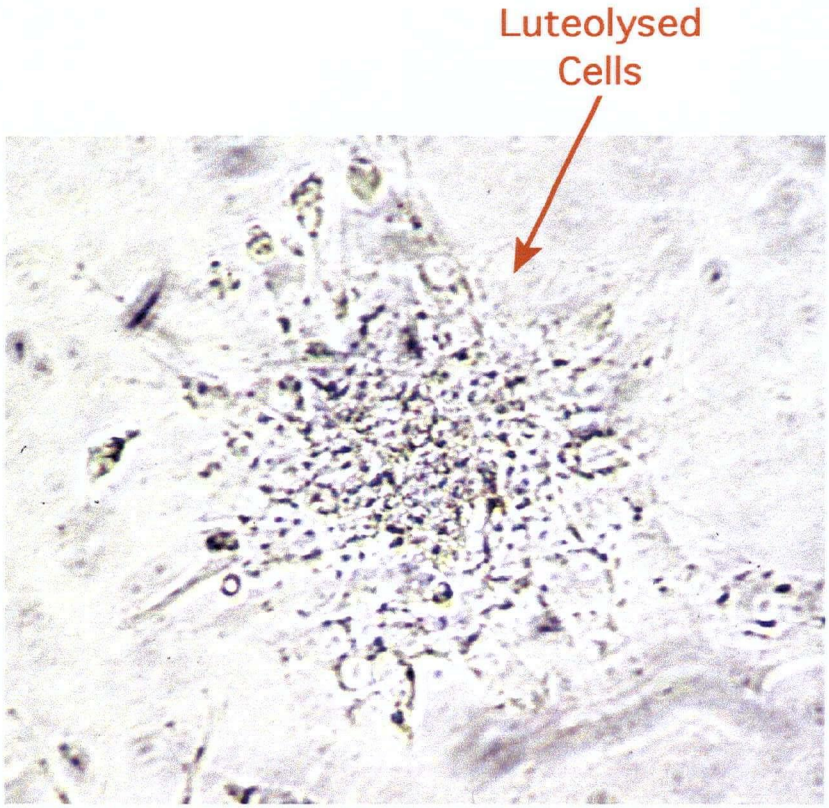


Figure 20. Sixteen day cultured human granulosa-luteal cells were no longer viable or responsive to stimuli and appeared to be luteolysed (66 x mag).

A. The Effects of $PGF_{2\alpha}$ on Steroidogenesis in the Absence and Presence of hCG

Effects of $PGF_{2\alpha}$ on Steroidogenesis

1. Progesterone and Estradiol Production in Response to $PGF_{2\alpha}$

Briefly, progesterone production in response to $PGF_{2\alpha}$ changed with culture time from inhibition (Fig. 21A) to stimulation (Fig. 21B; biphasic) in D_1 and $D_{12\text{-}14}$ cultured GLCs, respectively. While cells at D_8 of culture were in a state of transition with inhibition, stimulation or intermediate responses being possible (Fig. 22).

Progesterone production was significantly inhibited (50% of control; $a \neq c$, $p<0.001$) in a linear fashion by $PGF_{2\alpha}$ in D_1 cultured GLCs (Fig. 21A; n=4). Conversely, in $D_{12\text{-}14}$ cultured GLCs $PGF_{2\alpha}$ significantly stimulated progesterone production (Fig. 21B; 200% of control; $a \neq c$; $p<0.001$; n=5), with maximal stimulation at mid-range concentrations (10^{-8} to 10^{-10} M). Day_8 cultured GLCs were in a state of transition between D_1 and D_{14} cells, with four responses being present (Fig. 22; total n=9): inhibition (n=2), no response (not shown; n=1), linear stimulation (n=3) and bimodal stimulation (n=3).

In D_1 (n=6) and D_8 (n=5) pre-cultured human GLCs, $PGF_{2\alpha}$ had no effect and stimulated estradiol production, respectively (Fig. 23A). The stimulatory response was significant at low (10^{-12} to 10^{-8} M; $a \neq b$; $p<0.05$) and high concentrations of $PGF_{2\alpha}$ (10^{-7} to 10^{-6} M; $a \neq c$; $p<0.0001$).

2. DNA Levels in Response to $PGF_{2\alpha}$

DNA levels of GLC's remained unchanged by $PGF_{2\alpha}$ treatment in D_8 GLCs (Fig. 23B; n=3), suggesting that responses were due to alterations in steroid production rather than changes in the number of cells per well.

(see p. 121 for relevant discussion)

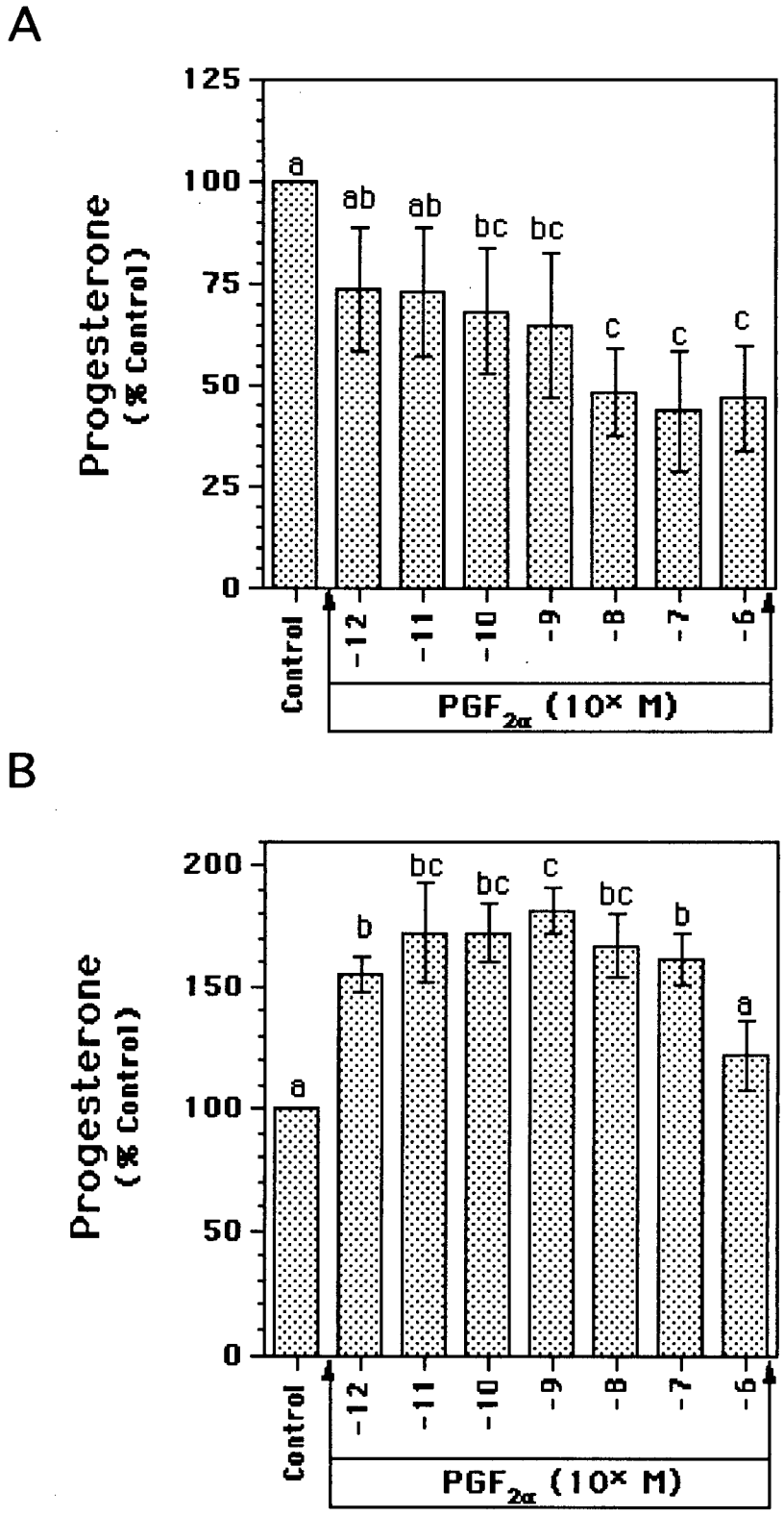


Figure 21. Progesterone production in response to $PGF_{2\alpha}$ treatment (for 24 h), in one-day (**A**; n=4; a≠c, p<0.001 by ANOVA) and twelve to fourteen-day (**B**; n=4; a≠c, p<0.001 by ANOVA) pre-cultured human granulosa-luteal cells (GLCs). In one and twelve to fourteen day pre-cultured human GLCs, $PGF_{2\alpha}$ inhibited and stimulated progesterone production, respectively.

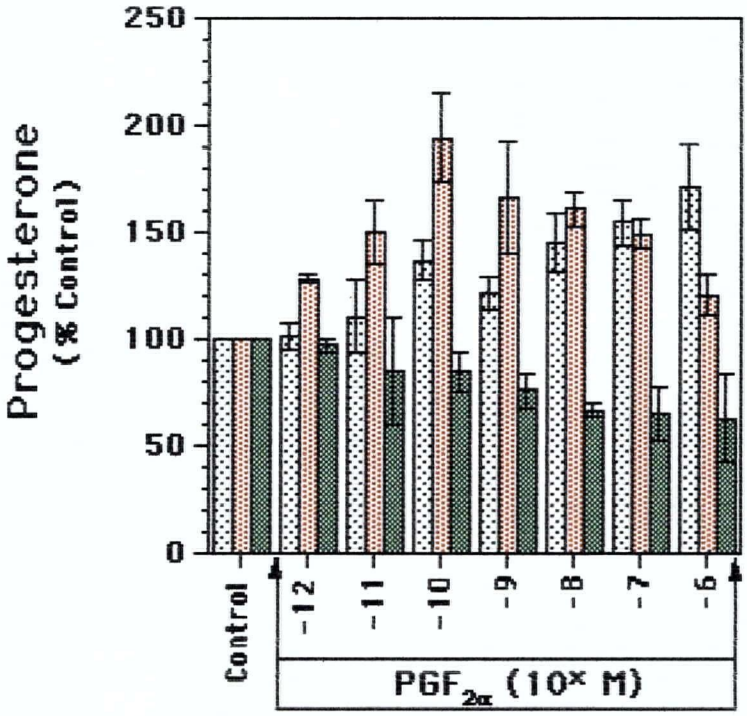


Figure 22. Progesterone production in response to $PGF_{2\alpha}$ treatment (for 24 h), in eight day pre-cultured human granulosa-luteal cells, four different progesterone-responses to $PGF_{2\alpha}$ were seen in nine separate experiments, including: linear stimulation (⬚; n=3), bell curve-like stimulation (▨; n=3), inhibition (▩; n=2) and no response (not shown; n=1).

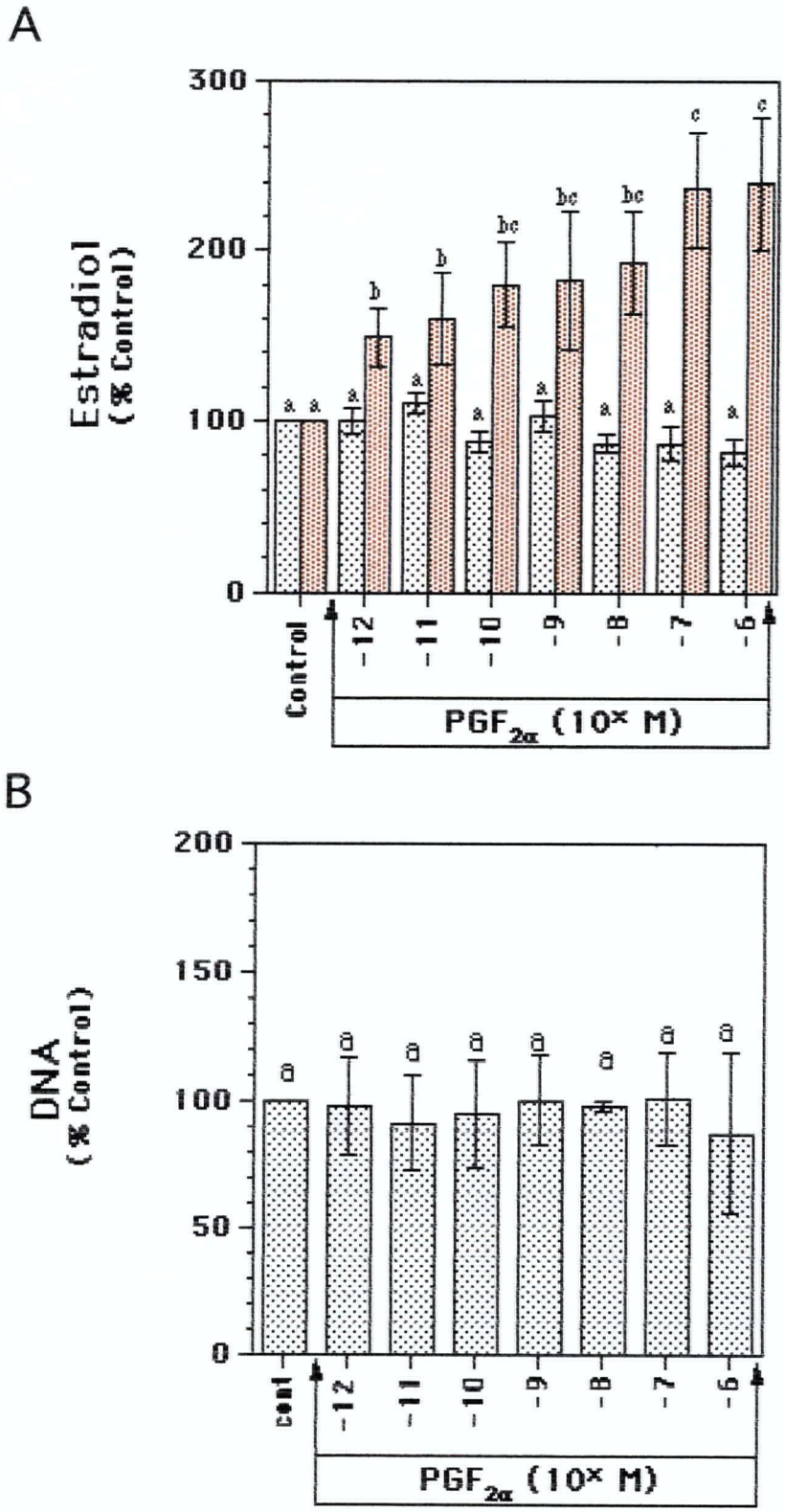


Figure 23. A. Estradiol production in response to $PGF_{2\alpha}$ treatment (for 24 h) in one (⬚; n=6)- and eight-day (▩; n=5; a≠b, $p<0.04$ and a≠c, $p<0.0001$ by ANOVA) pre-cultured human granulosa-luteal cells. DNA content (**B**; n=3) in response to $PGF_{2\alpha}$, in eight day pre-cultured human granulosa-luteal cells.

Effects of $PGF_{2\alpha}$ on hCG-Stimulated Steroidogenesis

1. Progesterone Production in Response to hCG Treatment

As shown in Figure 24, human chorionic gonadotrophin (0.001 to 10 IU/ml) significantly stimulated progesterone production up to six fold from human GLCs. The highest level of statistical significance was seen with 0.01 to 1 IU/ml (hCG) treated cells ($p<0.001$; n=4), However, cells treated with lower (0.001 IU/ml) and higher (10 IU/ml) concentrations of hCG still responded significantly ($p<0.05$).

2. Follicle Size-Dependent Regulation of Steroidogenesis by hCG and $PGF_{2\alpha}$

Cells from four patients were separated into small (< 12 mm) and large (> 12 mm)-follicle size groups and subjected to hCG (1 IU/ml) and hCG plus $PGF_{2\alpha}$ (10^{-6} M) treatment at D_1 (Fig. 25; n=4). Human chorionic gonadotrophin failed to significantly stimulate progesterone or estradiol production in GLCs collected from small follicles ($p>0.05$). In contrast, hCG stimulated progesterone ($p<0.001$) and estradiol ($p<0.02$) production in GLCs collected from large follicles. In addition, $PGF_{2\alpha}$ inhibited hCG-stimulated progesterone and estradiol production in GLCs from large follicles ($p<0.03$), while it did not in cells from small follicles.

3. The Effects of $PGF_{2\alpha}$ on hCG-Stimulated Steroidogenesis

In the presence of hCG, culture-time dependent changes in progesterone responses to $PGF_{2\alpha}$ were observed. Prostaglandin $F_{2\alpha}$ (10^{-6} M) inhibited hCG-stimulated progesterone production in D_1 (Fig. 26A; $p<0.05$; n=5) and D_8 (Fig. 26B; $p<0.01$; n=6), although not in D_{14} (Fig. 27; n=4) cultured GLCs. Alternately, $PGF_{2\alpha}$ (10^{-9} M) potentiated hCG-stimulated progesterone production in D_8 ($p<0.01$; n=6; 3 fold) and D_{14} ($p<0.05$; n=4; 1.5 fold), although not in D_1 (n=4) cultured GLCs.

A similar trend was seen with estradiol production. Prostaglandin $F_{2\alpha}$ (10^{-6} M) inhibited hCG-stimulated estradiol production in D_1 (Fig. 28A; $p<0.05$; n=8) and D_8 (Fig. 28B; $p<0.05$; n=5) cultured GLCs. Alternately, $PGF_{2\alpha}$ (10^{-9} M) potentiated hCG-stimulated estradiol production in D_8 ($p<0.01$; n=5; 1.5 fold), although not in D_1 (n=4) cultured GLCs.

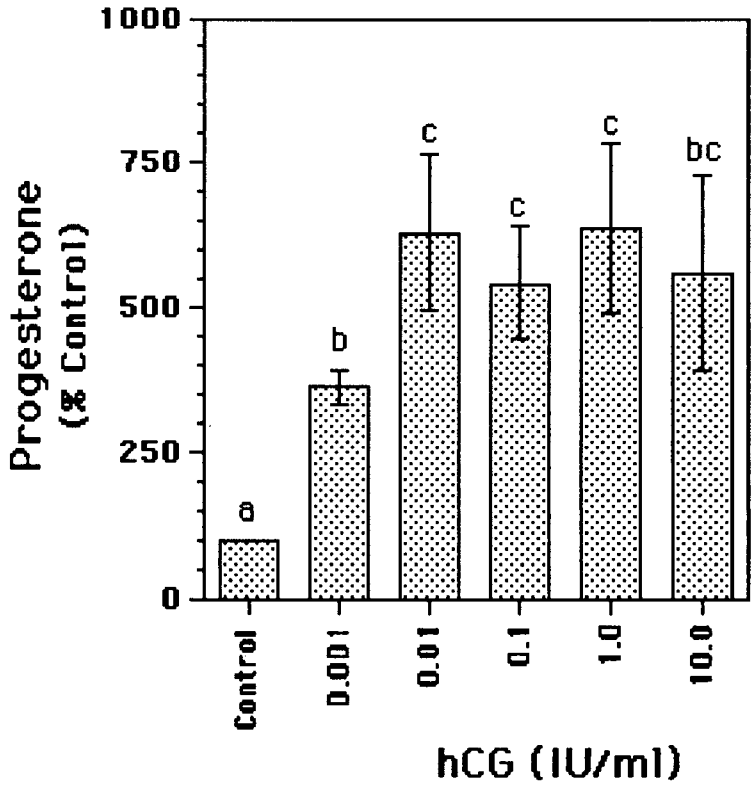


Figure 24. Human chorionic gonadotrophin (hCG) stimulated progesterone production from human granulosa-luteal cells treated for 24 h following eight days of culture (n=4; a≠b or b≠c, $p<0.05$; a≠c, $p<0.001$ by ANOVA).

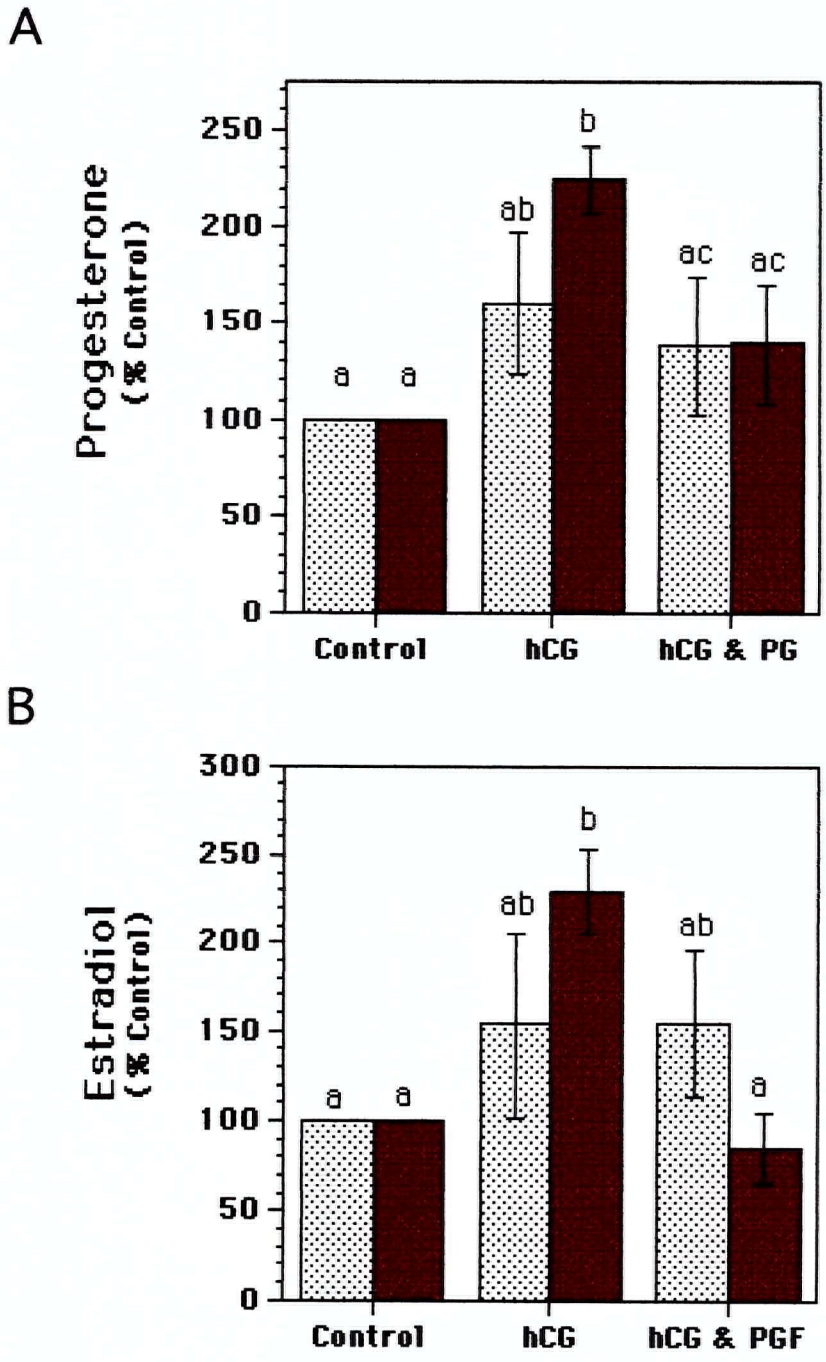


Figure 25. Follicle size-dependent responses to human chorionic gonadotrophin (hCG; 1 IU/ml) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment (for 24 h). **A.** In 1 day pre-cultured human granulosa-luteal cells, $PGF_{2\alpha}$ inhibited (n=4; a≠b, $p<0.001$ and b≠c, $p<0.03$ by ANOVA) hCG-stimulated progesterone production from cells collected from large follicles (■; > 12 mm). However, cells collected from small follicles (⊡; < 12 mm), were unable to respond to hCG. **B.** Similar results were seen for hCG-stimulated estradiol production (n=4; a≠b, $p<0.03$ by ANOVA).

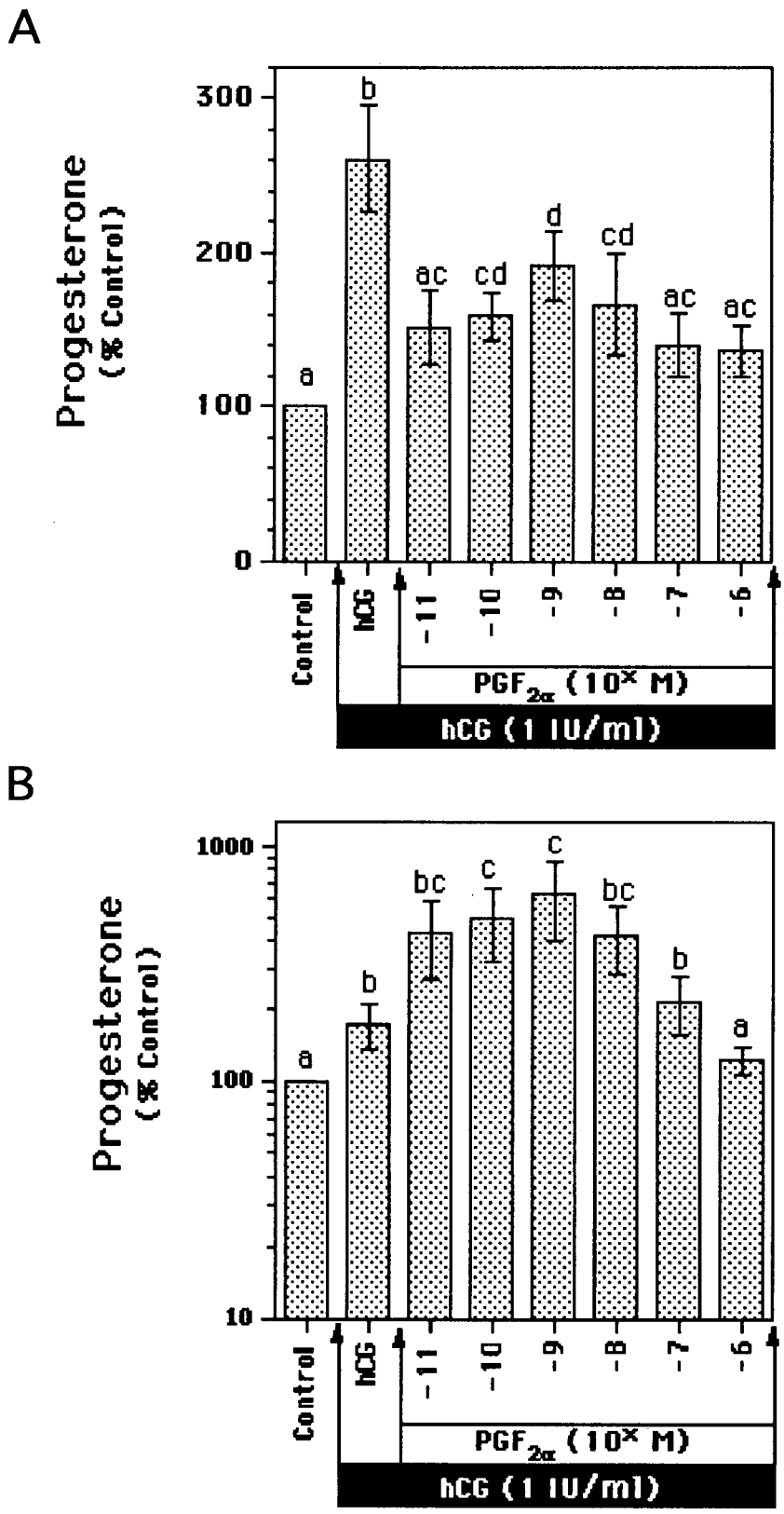


Figure 26. The effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on hCG-stimulated progesterone production (over 24 h) from one-day (**A**; n=5; a≠b≠c≠d, $p<0.05$ by ANOVA) and eight-day (**B**; n=6; a≠b≠c, $p<0.01$ by ANOVA) pre-cultured human granulosa-luteal cells.

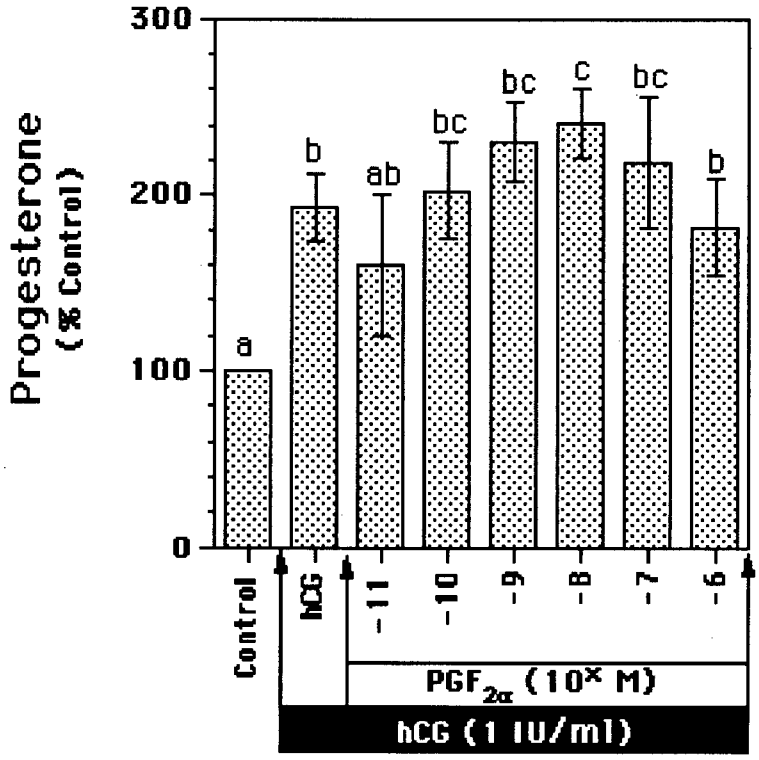


Figure 27. The effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on hCG-stimulated progesterone production (over 24 h) from twelve to fourteen-day (n=4; a≠b≠c, $p<0.05$ by ANOVA) pre-cultured human granulosa-luteal cells.

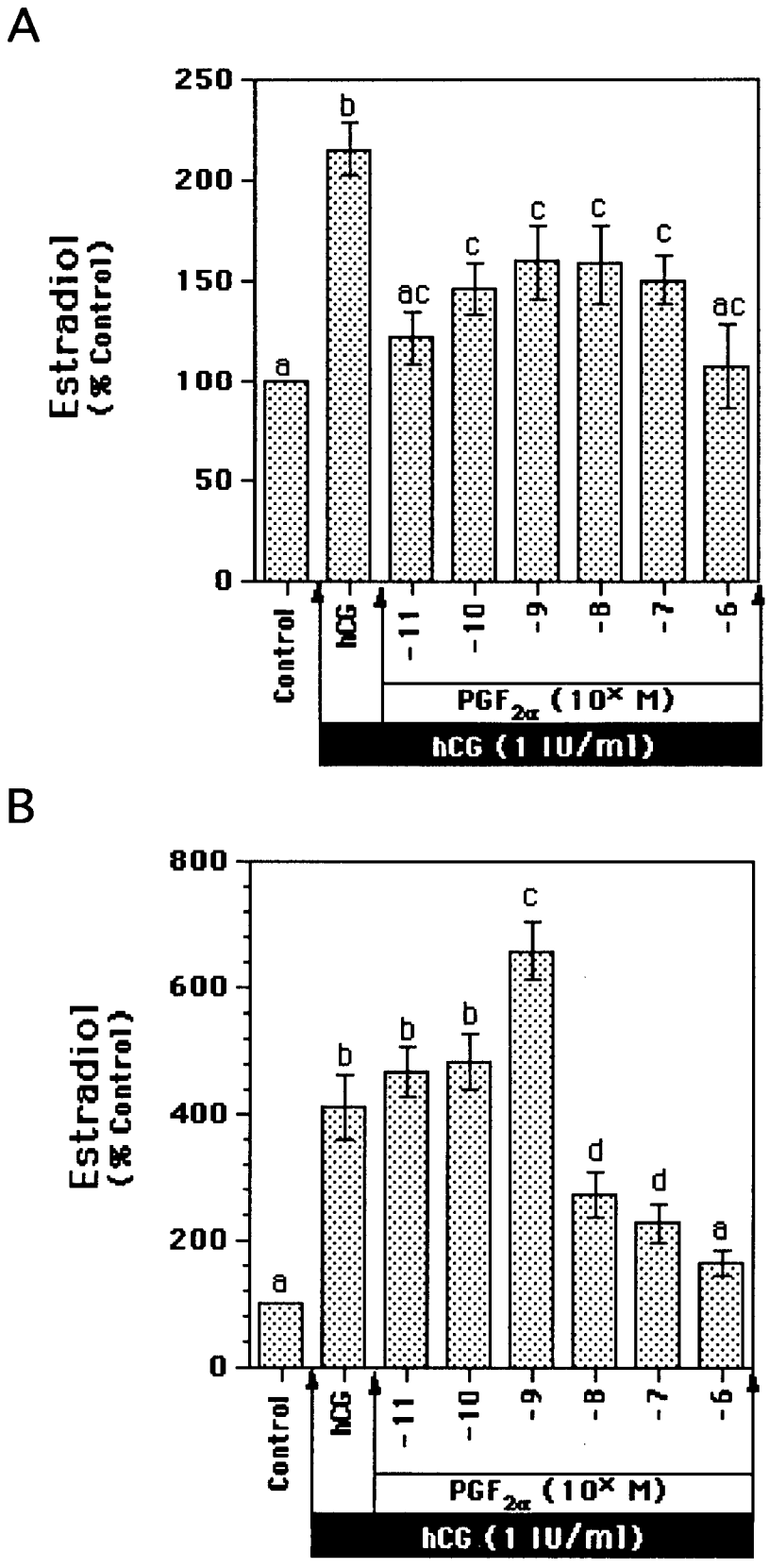


Figure 28. The effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on hCG-stimulated estradiol production (over 24 h) from one-day (**A**; n=8; a≠b≠c, $p<0.05$ by ANOVA) and eight-day (**B**; n=5; a≠b≠c, $p<0.05$ by ANOVA) pre-cultured human granulosa-luteal cells.

Effects of GnRH on hCG-Stimulated Steroidogenesis

Gonadotrophin-releasing hormone (10^{-6} M) inhibited hCG-stimulated progesterone production in D_1 (Fig. 29A; n=6; $p<0.05$) and D_8 (Fig. 29B; n=5; $p<0.05$) cultured GLCs. Alternately, GnRH (10^{-8} M) potentiated hCG-stimulated progesterone production in D_8 (Fig. 29B; n=5; $p<0.05$; $a \neq b \neq c$), although not in D_1 (n=6) cultured GLCs.

A similar trend was seen with estradiol production. Gonadotrophin-releasing hormone (10^{-6} M) inhibited hCG-stimulated estradiol production in D_8 (Fig. 30A; n=4; $p<0.05$) cultured GLCs. Alternately, GnRH (10^{-9} M) potentiated hCG-stimulated estradiol production in D_8 cultured GLCs (n=5; $p<0.01$). DNA levels were unaltered by any of the treatments (Fig. 30B; n=3; $p>0.05$).

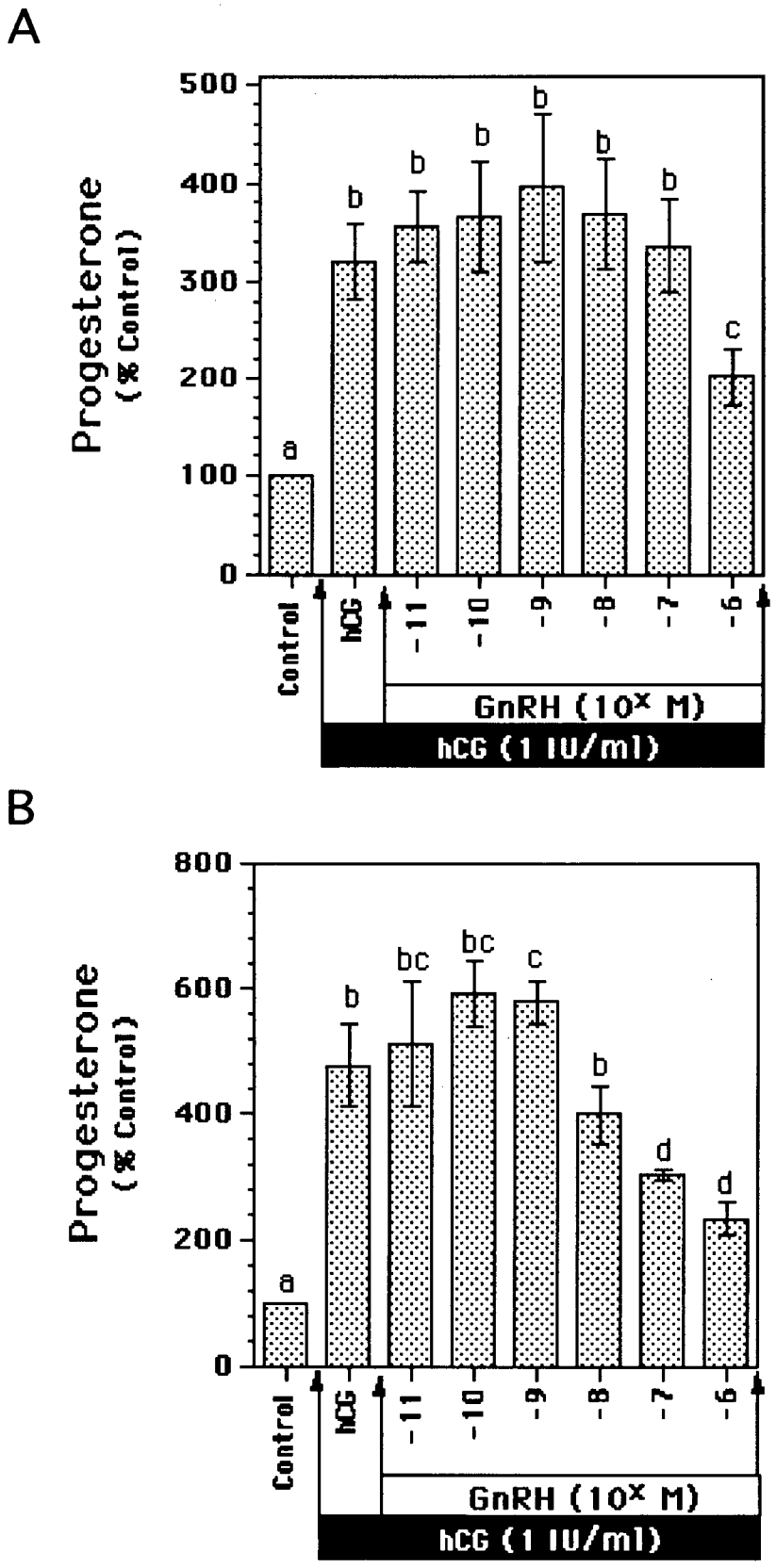


Figure 29. The effects of gonadotrophin-releasing hormone (GnRH) on hCG-stimulated progesterone production (over 24 h) from one-day (**A**; n=4; a≠b≠c, $p<0.05$ by ANOVA) and eight-day (**B**; n=5; a≠b≠c≠d, $p<0.05$ by ANOVA) pre-cultured human granulosa-luteal cells.

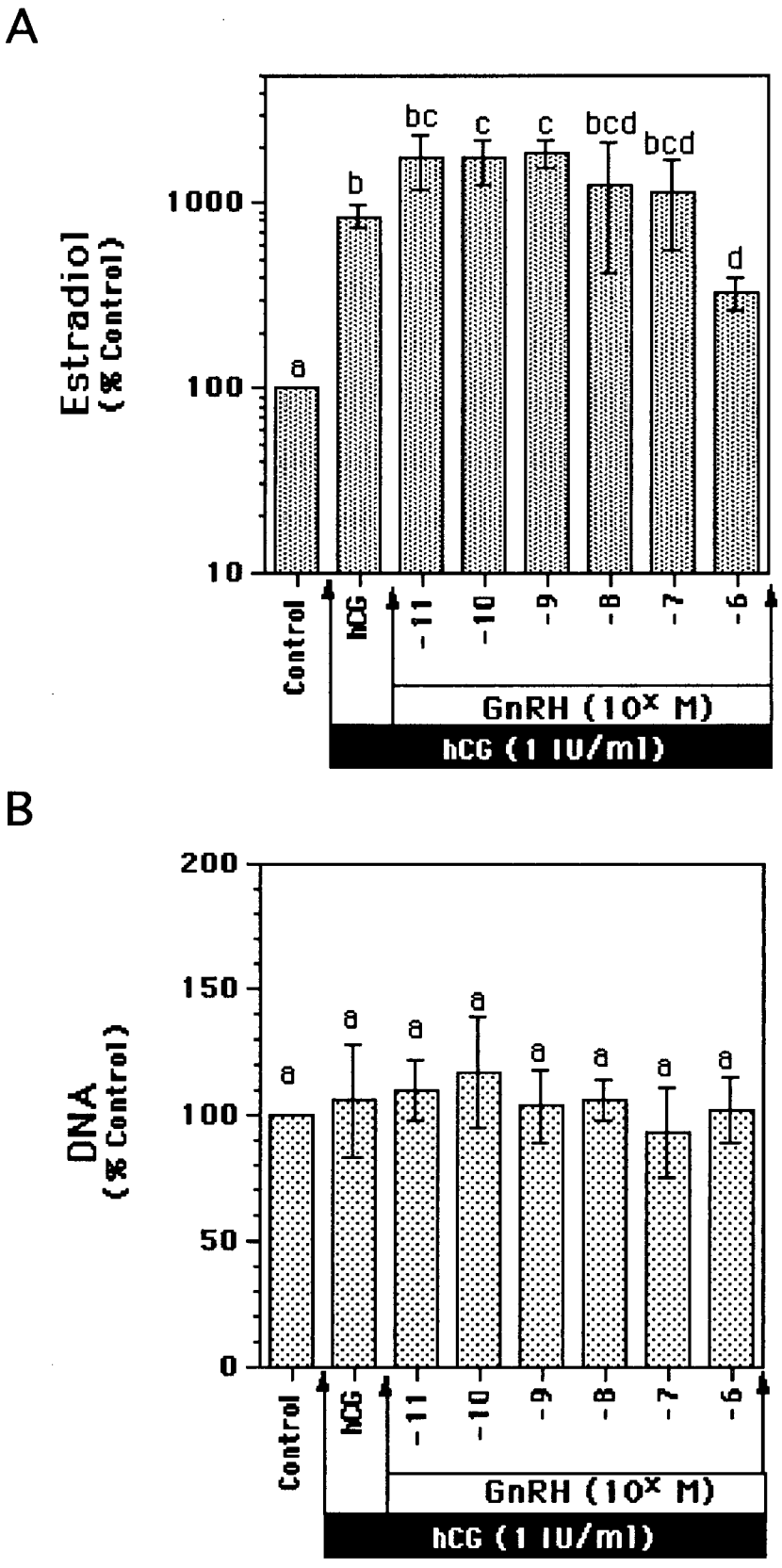


Figure 30. A. The effects of gonadotrophin-releasing hormone (GnRH) on hCG-stimulated estradiol production (over 24 h) from eight-day (n=4; a≠b≠c, $p<0.05$ by ANOVA) pre-cultured human granulosa-luteal cells. **B**. DNA levels were unaltered by any of the above treatments (n=2; $p>0.05$ by ANOVA).

B. The Interaction of $PGF_{2\alpha}$ with GnRH

Progesterone Response to GnRH and/or $PGF_{2\alpha}$, with or without hCG.

Neither $PGF_{2\alpha}$ (10^{-9} M) nor GnRH (10^{-6} M) significantly altered progesterone production, in D_1 human GLCs (Fig. 31A; n=5). However, the combination of $PGF_{2\alpha}$ plus GnRH significantly stimulated progesterone production ($a \neq b$, $p<0.05$). Human chorionic gonadotrophin (1 IU/ml) also significantly stimulated progesterone production (2.5-3 fold; $a \neq b$, $p<0.05$). Conversely, gonadotrophin-releasing hormone alone was unable to inhibit hCG-stimulated progesterone production, although it did potentiate $PGF_{2\alpha}$-mediated inhibition ($a \neq b \neq c$, $p<0.05$).

In D_8 pre-cultured GLCs a significant luteotrophic response to $PGF_{2\alpha}$ (10^{-9} M) was present (Fig. 31B; n=4; $p<0.05$). However, no luteotrophic response to GnRH (10^{-6} M) was observed, although GnRH potentiated the $PGF_{2\alpha}$-mediated luteotrophic response ($p<0.05$). Both GnRH and $PGF_{2\alpha}$ significantly inhibited hCG-stimulated progesterone production ($b \neq c$, $p<0.05$), while their combination potentiated inhibition beyond levels of either hormone alone ($p<0.05$).

Estradiol Response to GnRH and/or $PGF_{2\alpha}$, with or without hCG.

In D_1 human granulosa luteal cells (Fig. 32; n=3), neither $PGF_{2\alpha}$ (10^{-9} M) nor GnRH (10^{-6} M) significantly altered estradiol production. However, the combination of $PGF_{2\alpha}$ plus GnRH significantly stimulated estradiol production ($p<0.01$). Human chorionic gonadotrophin (1 IU/ml) also significantly stimulated estradiol production ($p<0.05$). Gonadotrophin-releasing hormone alone was unable to inhibit hCG-stimulated progesterone production, although it did potentiate $PGF_{2\alpha}$-mediated inhibition ($p<0.05$).

Progesterone Response to GnRH with or without $PGF_{2\alpha}$

Vehicle, $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M) and GnRH (10^{-10} to 10^{-5} M) concentration-response curves were crossed into a matrix of 49 separate treatments. Results were plotted in three dimensions, with GnRH, $PGF_{2\alpha}$ and progesterone-response each on one axis (Fig. 33A and B

[mirror image of A]). Results were also plotted and as a contour map with GnRH and $PGF_{2\alpha}$ each on a separate axis and progesterone response represented by shading (Fig. 34A) and colour (Fig. 34B). In D_1 human GLCs, maximal stimulation of progesterone-production (2-3 fold) was seen when middle concentrations of $PGF_{2\alpha}$ (10^{-9} M; $p<0.05$) interacted with high concentrations of GnRH (10^{-6} to 10^{-5} M). In the presence of high concentrations of GnRH (10^{-6} M), $PGF_{2\alpha}$ stimulated progesterone production in a bell curve-like fashion as middle concentrations significantly stimulated while low and high concentrations did not (Fig. 35; $p<0.05$). In the presence of middle concentrations of $PGF_{2\alpha}$ (10^{-9} M), GnRH significantly stimulated progesterone-production in a linear concentration-dependent manner (Fig. 36; $p<0.05$).

Estradiol Response to GnRH with or without $PGF_{2\alpha}$

In D_1 human GLCs, maximal stimulation of estradiol-production (4-fold) was seen when high concentrations of $PGF_{2\alpha}$ (10^{-6} M; $p<0.05$) interacted with high concentrations of GnRH (10^{-6} to 10^{-5} M). These data are presented in three dimensional graph (Fig. 37; n=6) and contour format (Fig. 38), as for progesterone data above. In the presence of high concentrations of GnRH (Fig. 39; 10^{-5} M), $PGF_{2\alpha}$ significantly and linearly stimulated estradiol production ($p<0.05$), although $PGF_{2\alpha}$ was ineffective in the absence of GnRH. On the other hand, in the absence and presence of $PGF_{2\alpha}$ (10^{-6} M), GnRH significantly stimulated estradiol production (Fig. 40A and B; $p<0.05$). The nature of GnRH stimulated estradiol production was, however, different in the presence and absence of $PGF_{2\alpha}$, as the response shifted from a bell curve-like stimulation to a linear one with the addition of $PGF_{2\alpha}$.

DNA Levels in Response to GnRH and $PGF_{2\alpha}$ Treatment

DNA Levels were unaltered by treatment with either GnRH (10^{-10} to 10^{-5} M), $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M) or hCG, suggesting that responses seen were due to alterations in steroid production rather than changes in cell numbers (data not shown).

Effects of Indomethacin on $PGF_{2\alpha}$ and GnRH Stimulated Steroidogenesis

In D_8 cultured human GLCs, progesterone was significantly stimulated in a bell curve-like fashion by $PGF_{2\alpha}$ (Fig. 41). Maximal stimulation of progesterone production was at 1 nM of $PGF_{2\alpha}$ ($p<0.05$). However, co-incubation with indomethacin (10^{-6} M) reversed this effect, and $PGF_{2\alpha}$ instead inhibited or had no effect on progesterone production (depending on concentration). Similar results were seen in cells from two other patients. Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude tryphan blue.

Progesterone production in D_1 cultured human GLCs was uneffected ($p>0.05$) by vehicle, GnRH (10^{-10} to 10^{-6} M) and/or $PGF_{2\alpha}$ (10^{-11} to 10^{-6}) when cells were co-incubated with indomethacin (10^{-6} M; n=4; Fig. 42). Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude tryphan blue. Compare these results and those presented in Figure 33. Note that hCG-stimulated progesterone production was seen in these cells (not shown).

(see p. 125 for relevant discussion)

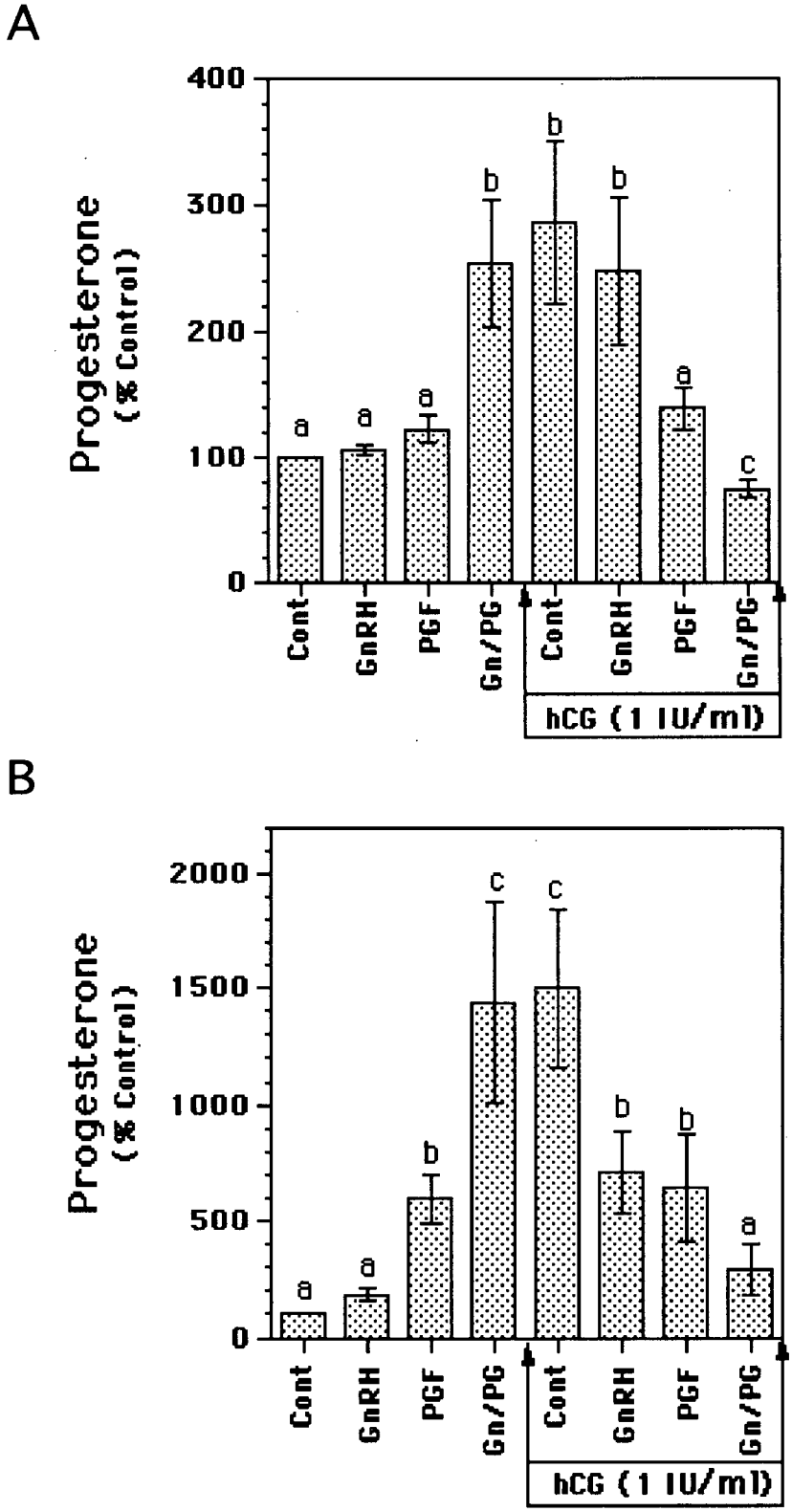


Figure 31. Progesterone production in response to vehicle (Cont), gonadotrophin-releasing hormone (GnRH; 10^{-6} M), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-9} M) and GnRH plus $PGF_{2\alpha}$ treatment (over 24 h), in the presence and absence of human chorionic gonadotrophin (hCG), in one-day (**A**; n=5) and eight-day (**B**; n=4) pre-cultured human granulosa-luteal cells. Graph bars represent mean ± SEM of experiments performed on separate patients ($a \neq b \neq c$, $p<0.05$ by ANOVA).

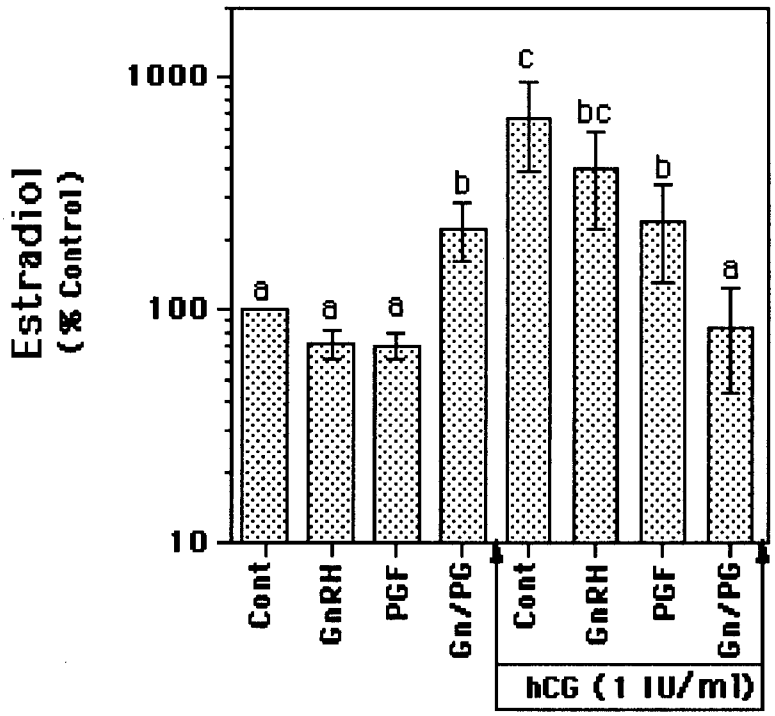


Figure 32. Estradiol production in response to vehicle (Cont), gonadotrophin-releasing hormone (GnRH; 10^{-6} M), prostaglandin $F_{2\alpha}$ (PGF; 10^{-9} M) and GnRH plus PGF treatment (over 24 h), in the presence and absence of human chorionic gonadotrophin (hCG), in one day (n=3) pre-cultured human granulosa-luteal cells. Graph bars represent mean ± SEM of experiments performed on separate patients ($a \neq b \neq c$, $p<0.05$ by ANOVA).

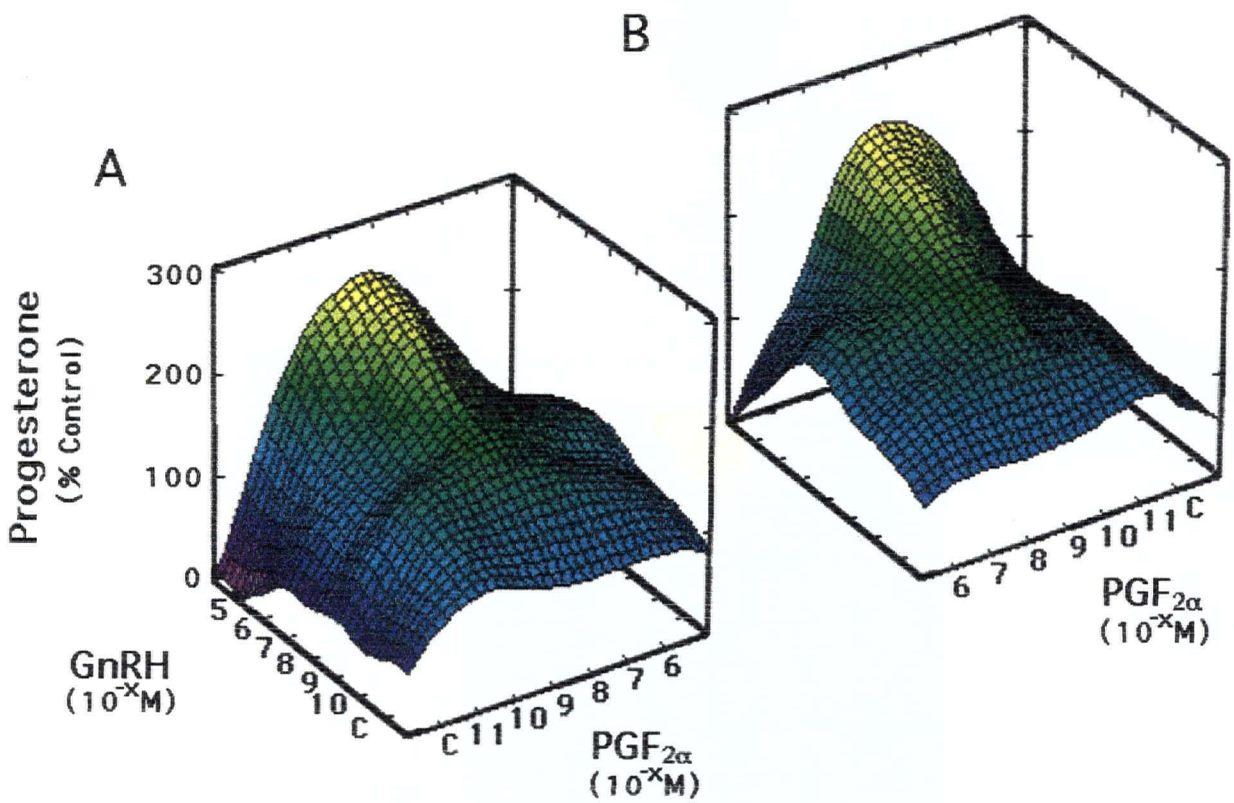


Figure 33. Three dimensional plot of progesterone production in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; 10^{-10} to 10^{-5} M) and/or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. A mirror image (**B**) provides a view of the back side of the image (**A**). These Figures represent the mean of seven separate experiments performed on seven separate patients.

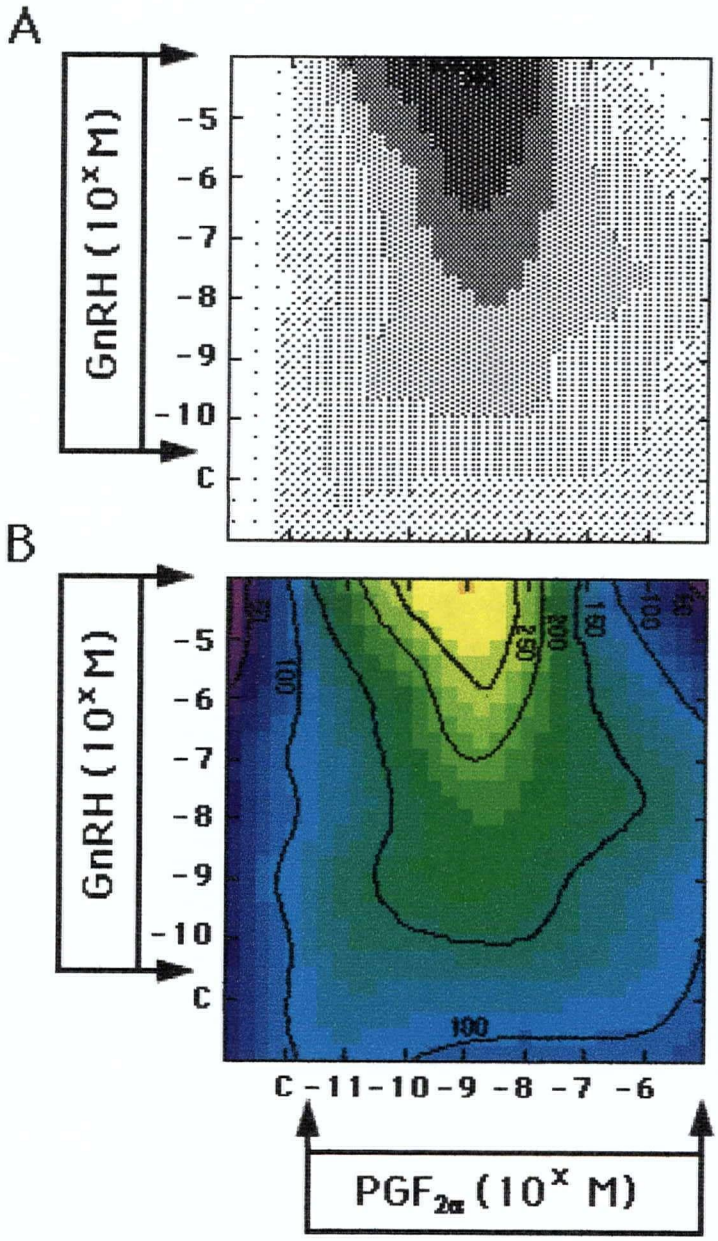


Figure 34. Black and white (**A**) and colour (**B**) contour plot of progesterone production in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; 10^{-10} to 10^{-5} M) and/or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. Progesterone production of 50, 100, 200 and 300% of the control level are symbolized by: ☐■, ⁙■, ▒■, and ▓■, respectively. These Figures represent the mean of seven separate experiments performed on seven separate patients.

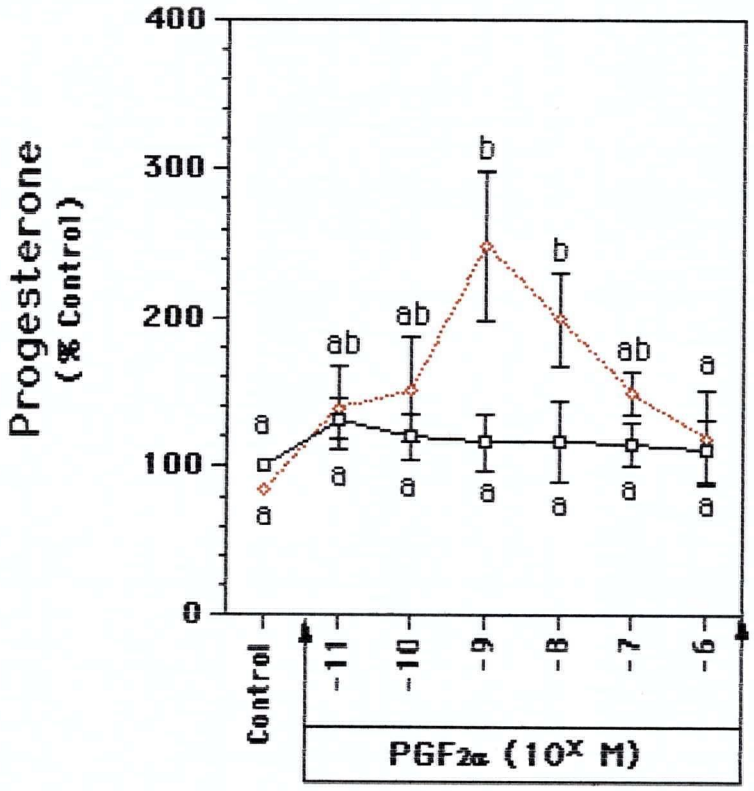


Figure 35. Effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M), in the absence (—□—) and presence (···◇···) of gonadotrophin-releasing hormone (GnRH; 10^{-6} M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. In the presence of GnRH, $PGF_{2\alpha}$ stimulated progesterone production in a bell curve-like fashion, with significant stimulation at middle concentrations (10^{-9} and 10^{-8} M $PGF_{2\alpha}$; a≠b, $p<0.05$, by ANOVA). While in the absence of GnRH, $PGF_{2\alpha}$ did not significantly alter progesterone production. Progesterone production in response to GnRH alone (10^{-6} M) was not significantly different from the control response. This Figure represents the mean ± sem of seven separate experiments performed on seven separate patients, and is a two dimensional slice of three dimensional matrix presented in Figure 33A.

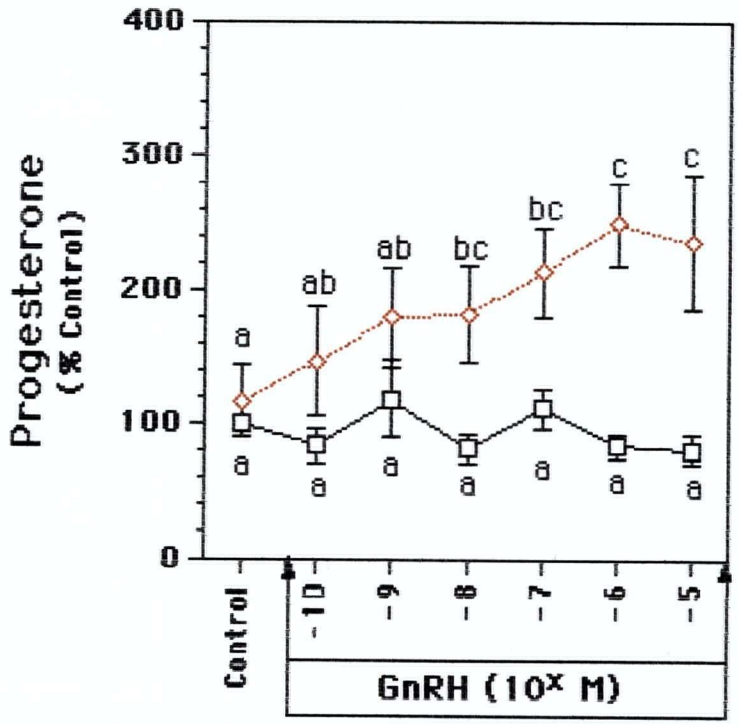


Figure 36. Effects of gonadotrophin-releasing hormone (GnRH; 10^{-10} to 10^{-5} M), in the absence (—□—) and presence (···◇···) of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-9} M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. In the presence of $PGF_{2\alpha}$, GnRH stimulated progesterone production in a linear concentration-dependent fashion, with significant stimulation at upper concentrations (10^{-8} to 10^{-5} M; a≠b, $p<0.05$ by ANOVA). While in the absence of $PGF_{2\alpha}$, GnRH had no effect on progesterone production. Progesterone production in response to $PGF_{2\alpha}$ alone (10^{-9} M) was not significantly different from the control response. This Figure represents the mean ± SEM of seven separate experiments performed on seven separate patients, and is a two dimensional slice of three dimensional matrix presented in Figure 33A

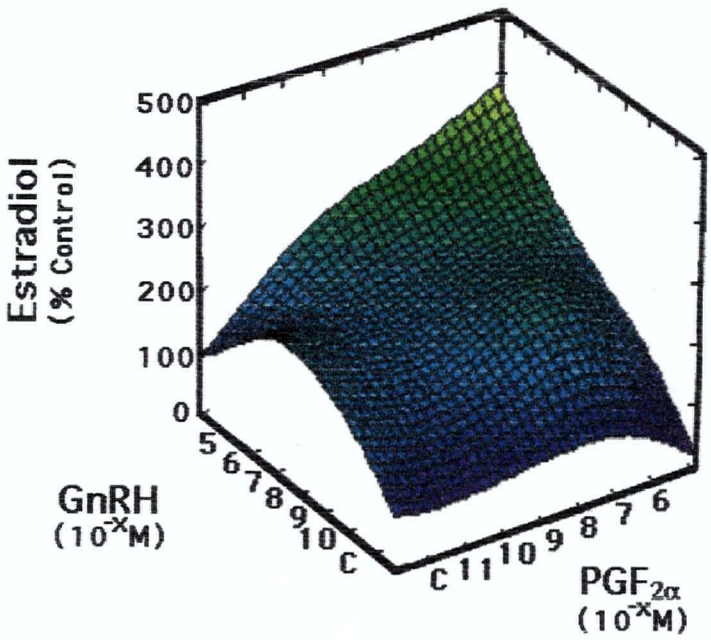


Figure 37. Three dimensional plot of estradiol production (over 24 h) in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; 10^{-10} to 10^{-5} M) and/or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M), in one day pre-cultured human granulosa-luteal cells. This Figure represents the mean of six separate experiments performed on six separate patients.

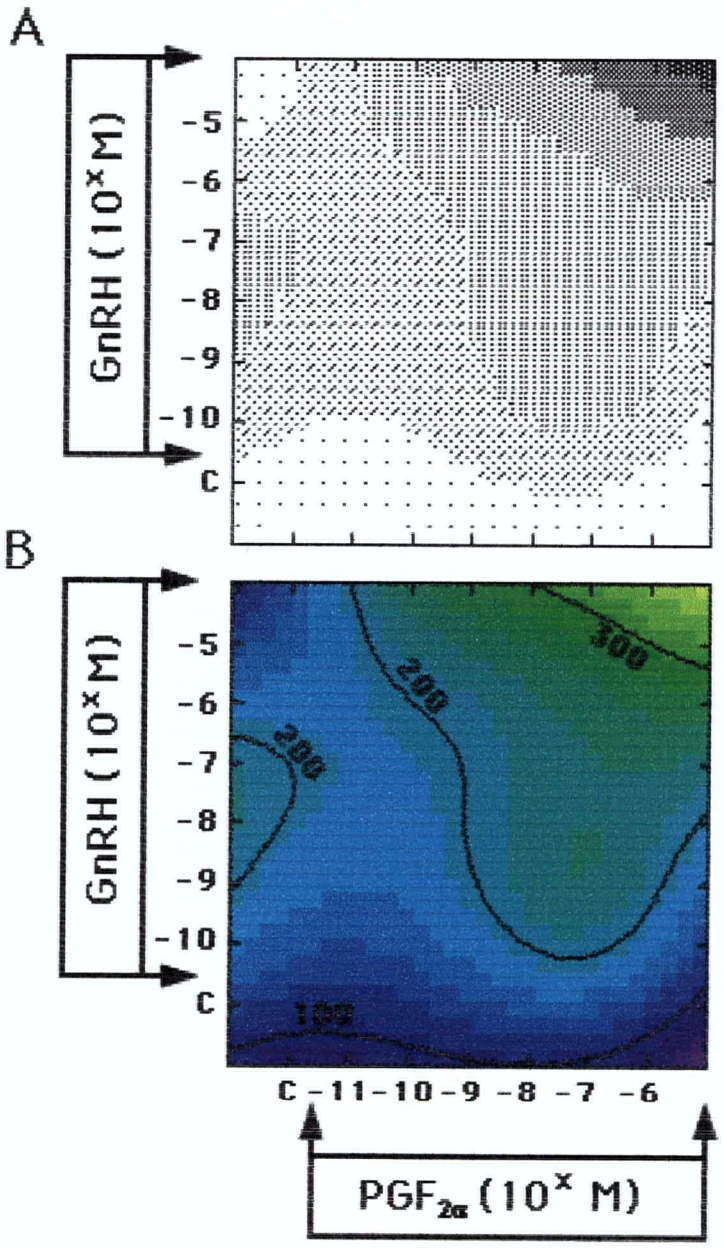


Figure 38. Black and white (**A**) and colour (**B**) contour plot of estradiol production (over 24 h) in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; 10^{-10} to 10^{-5} M) and/or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M), in one day pre-cultured human granulosa-luteal cells. Estradiol production of 100, 200, 300 and 400% of the control level are symbolized by: ■■, ■■, ■■ and ■■, respectively. These Figures represent the mean of six separate experiments performed on six separate patients.

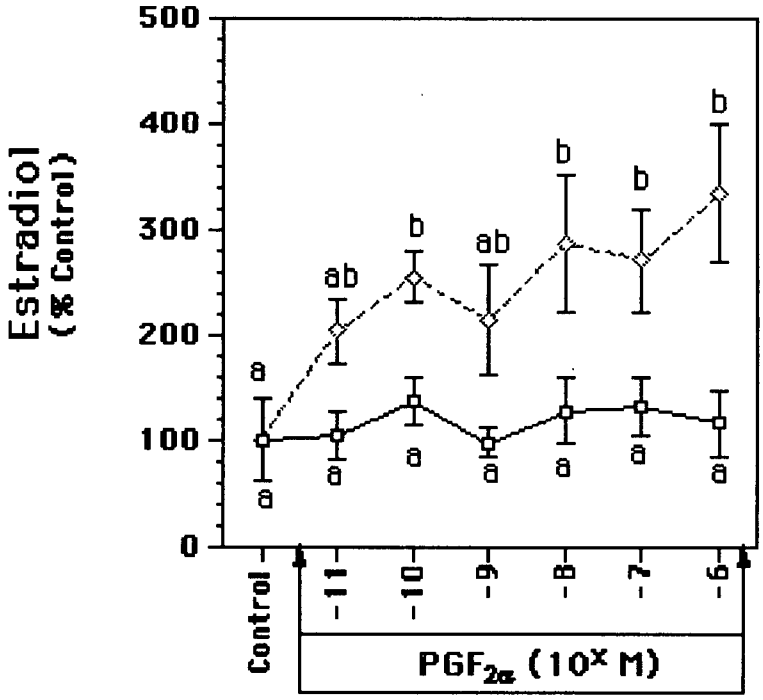


Figure 39. The interaction of gonadotrophin-releasing hormone (GnRH; 10^{-5} M) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on estradiol response (over 24 h), in one day pre-cultured human granulosa-luteal cells. In the presence (---◇---) of GnRH (10^{-5} M), $PGF_{2\alpha}$ significantly stimulated estradiol production (n=6; a≠b, $p<0.05$ by ANOVA), however $PGF_{2\alpha}$ was ineffective in the absence (—□—) of GnRH. These data represent a two dimensional slice of the three dimensional graph presented in Figure 37.

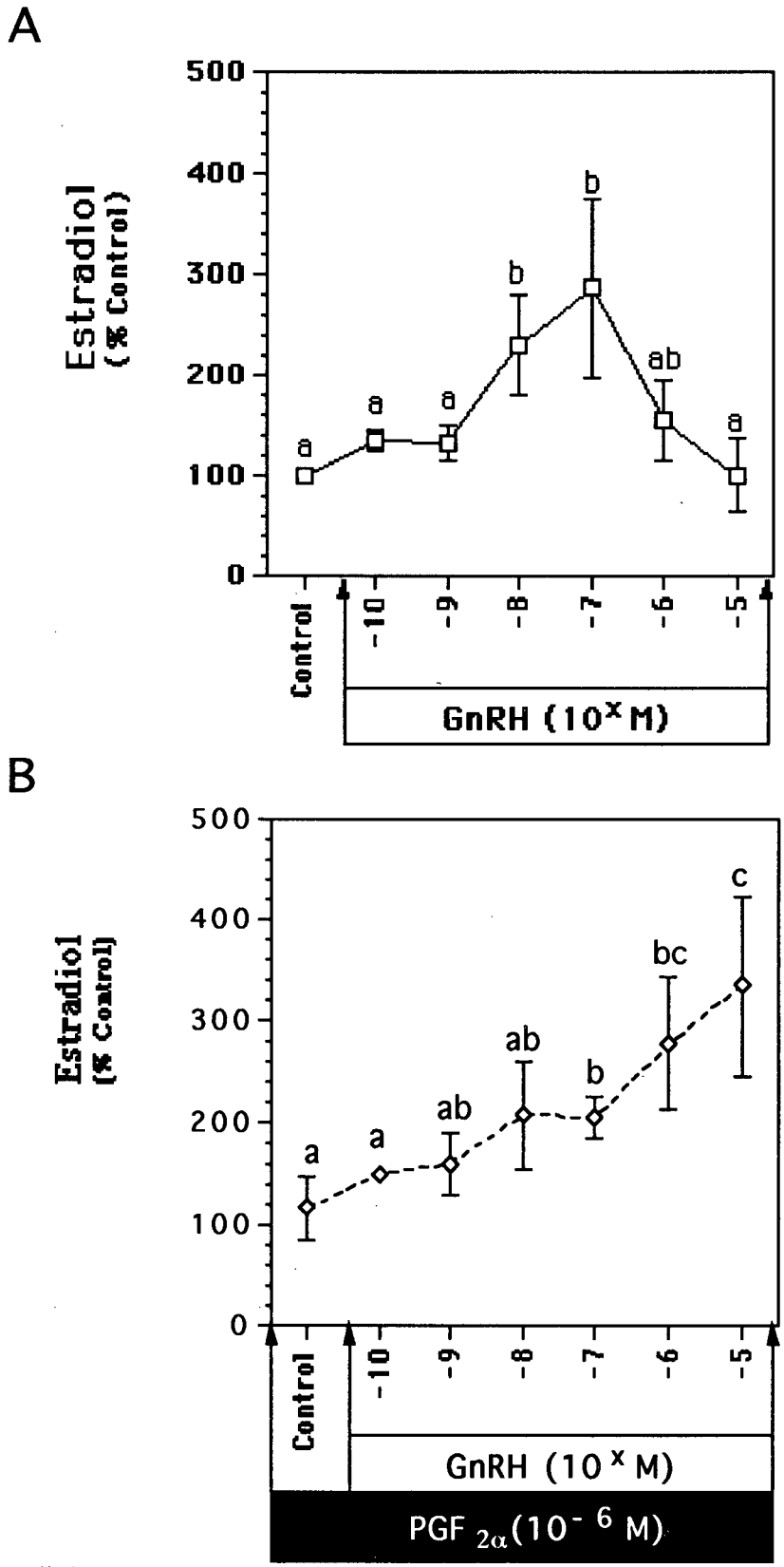


Figure 40. Estradiol response (over 24 h) to gonadotrophin-releasing hormone (GnRH; 10^{-5} M) in the absence (A) and presence (B) of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M), in one day pre-cultured human granulosa-luteal cells. In the presence of $PGF_{2\alpha}$, GnRH significantly stimulated estradiol production (n=6; a≠b≠c, $p<0.05$ by ANOVA). These data represent a 2D slice those in Figure 37.

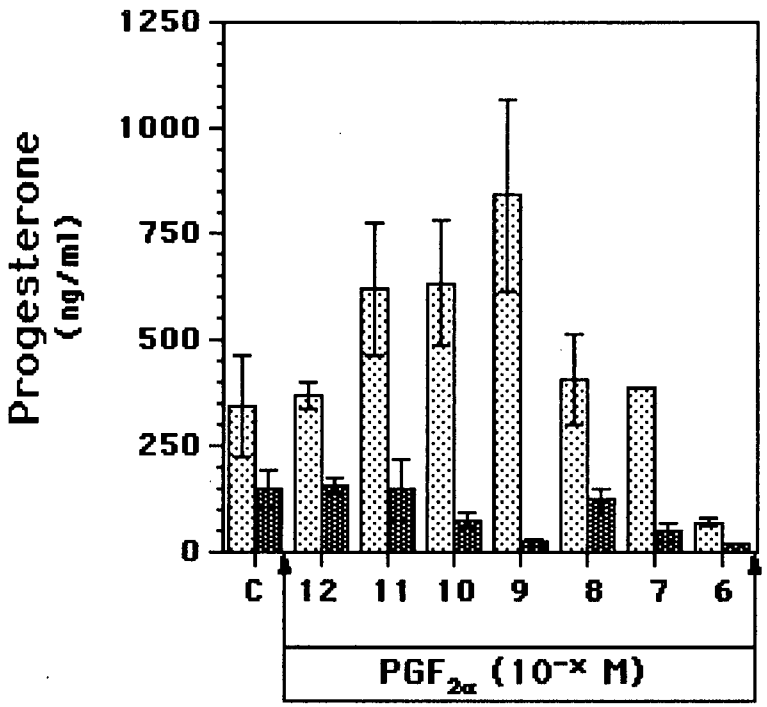


Figure 41. Progesterone response (over 24 h) to $PGF_{2\alpha}$ in the absence (▒) and presence (▓) of indomethacin (10^{-6} M), in eight day pre-cultured human granulosa-luteal cells. In the absence of indomethacin, $PGF_{2\alpha}$ significantly and in a bell curve-like fashion stimulated progesterone production ($p<0.05$; control vs $PGF_{2\alpha}$, 10^{-9} M). However, in the presence of indomethacin, $PGF_{2\alpha}$ either inhibited ($p<0.05$; control vs $PGF_{2\alpha}$, 10^{-10} to 10^{-6} M) or had no effect on progesterone production. This Figure represents the response of cells from one patient. Similar results were seen in cells from two other patients. Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude tryphan blue.

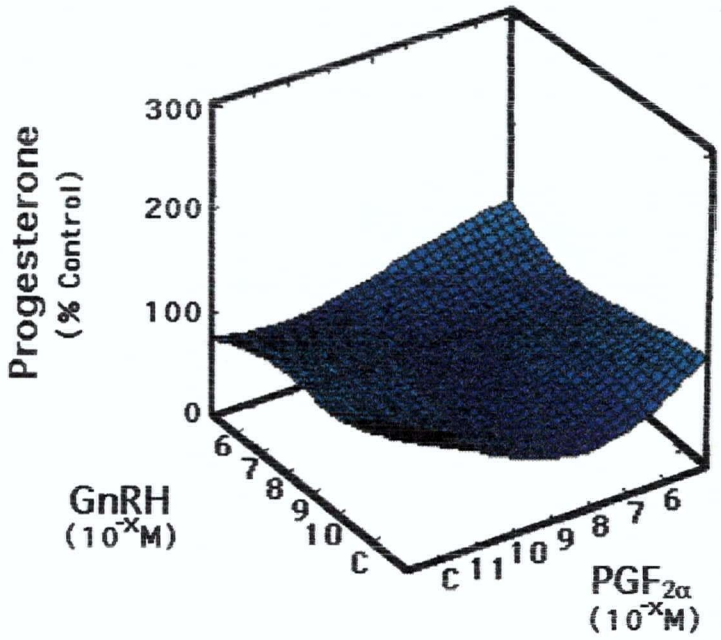


Figure 42. Three dimensional plot of progesterone production (over 24 h) in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; 10^{-10} to 10^{-6} M) and/or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M) in the presence of indomethacin (10^{-6} M), in one day pre-cultured human granulosa-luteal cells. This Figure represents the mean of four separate experiments performed on cells from four separate patients. No significant difference between treatments was seen ($p>0.05$). Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude tryphan blue.

C. Progesterone Response to $PGF_{2\alpha}$ plus PGE_2

The following study reveals a complex regulation of progesterone production in response to vehicle $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M) and/or PGE_2 (10^{-11} to 10^{-6} M) concentration-response curves in eight day cultured human GLCs. Prostaglandin $F_{2\alpha}$ and PGE_2 concentration-response curves were crossed into a matrix of 49 separate treatments. Results were plotted in three dimensions with $PGF_{2\alpha}$, PGE_2 and progesterone-response each on a separate axis (Fig. 43). Additionally, data were plotted in contour map form (Fig. 44). Moreover, two dimensional slices of the three dimensional matrix were plotted and analyzed statistically (Fig. 45). Briefly, progesterone was significantly stimulated in a bell curve-like manner by $PGF_{2\alpha}$ with maximal stimulation at 1 nM ($p<0.05$). A similar response to PGE_2 was seen although the bell curve was shifted right. Maximal PGE_2-mediated stimulation of progesterone production was seen at 10 to 100 nM (Fig. 45). However, in the presence of PGE_2 (10^{-7} M), $PGF_{2\alpha}$ significantly inhibited progesterone production ($p<0.05$) in an inverse bell curve-like manner, with maximal inhibition at (10^{-10} to 10^{-8} M, $PGF_{2\alpha}$; Fig. 46).

(see p. 131 for relevant discussion)

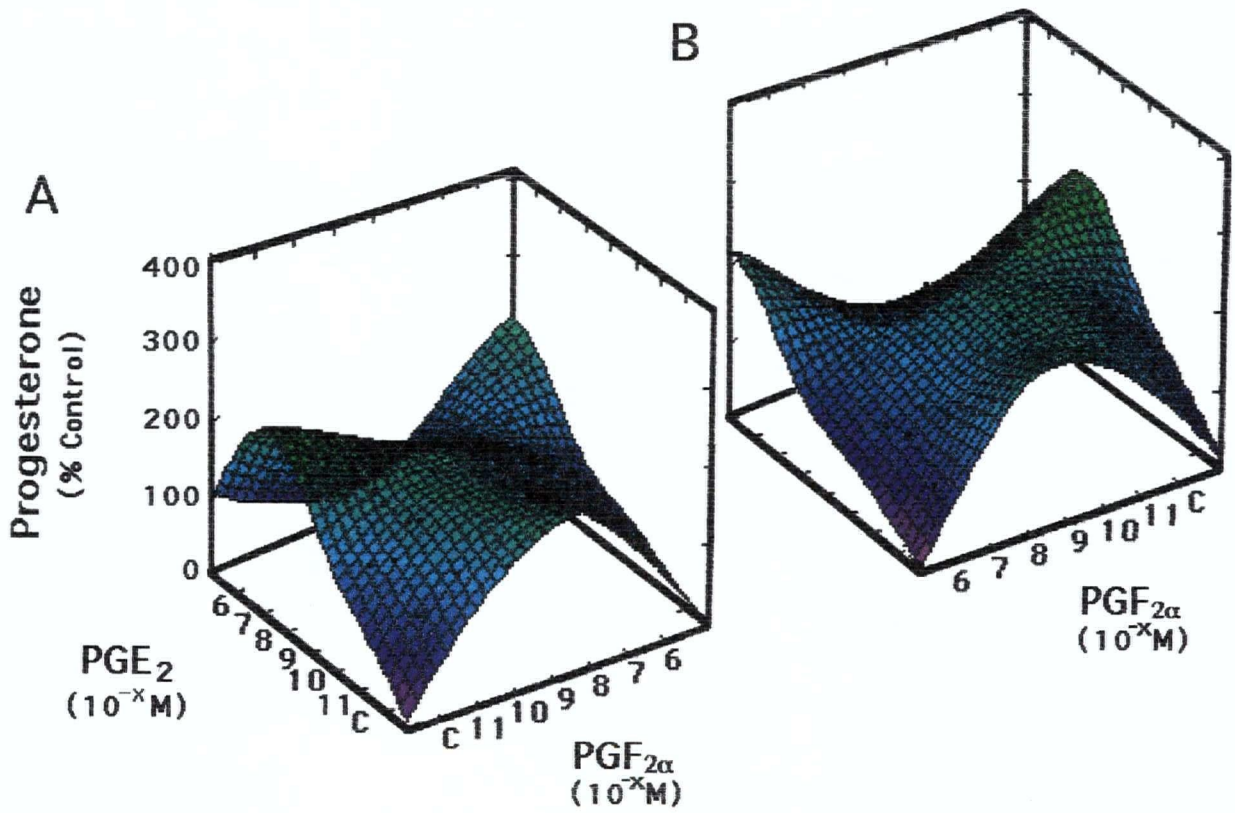


Figure 43. Progesterone production (over 24 h) in response to vehicle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M) and/or prostaglandin E_2 (PGE_2; 10^{-11} to 10^{-6} M) concentration response curves, in eight day pre-cultured human granulosa-luteal cells. A mirror image (**B**) provides a view of the back side of the image (**A**). This Figure represents the mean of four separate experiments performed on cells from four different patients.

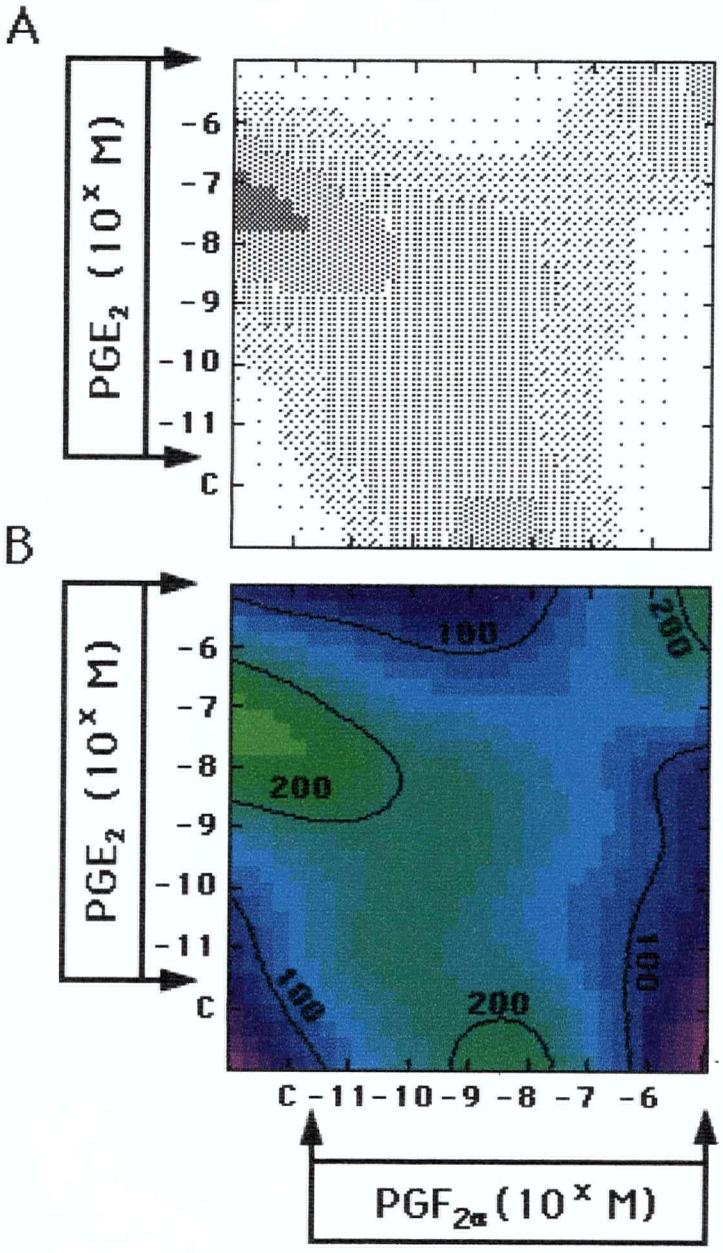


Figure 44. Black and white (**A**) and colour (**B**) contour plots of progesterone production (over 24 h) in response to vehicle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M) and/or prostaglandin E_2 (PGE_2; 10^{-11} to 10^{-6} M) concentration response curves (n=4), in eight day pre-cultured human granulosa-luteal cells. Progesterone production of 100, 200, and 300% of control level are symbolized by: □ ■, ▨ ■, and ▩ ■, respectively.This Figure represents the mean of four separate experiments performed on cells from four different patients, and is derived from the same data as those presented in Figure 43.

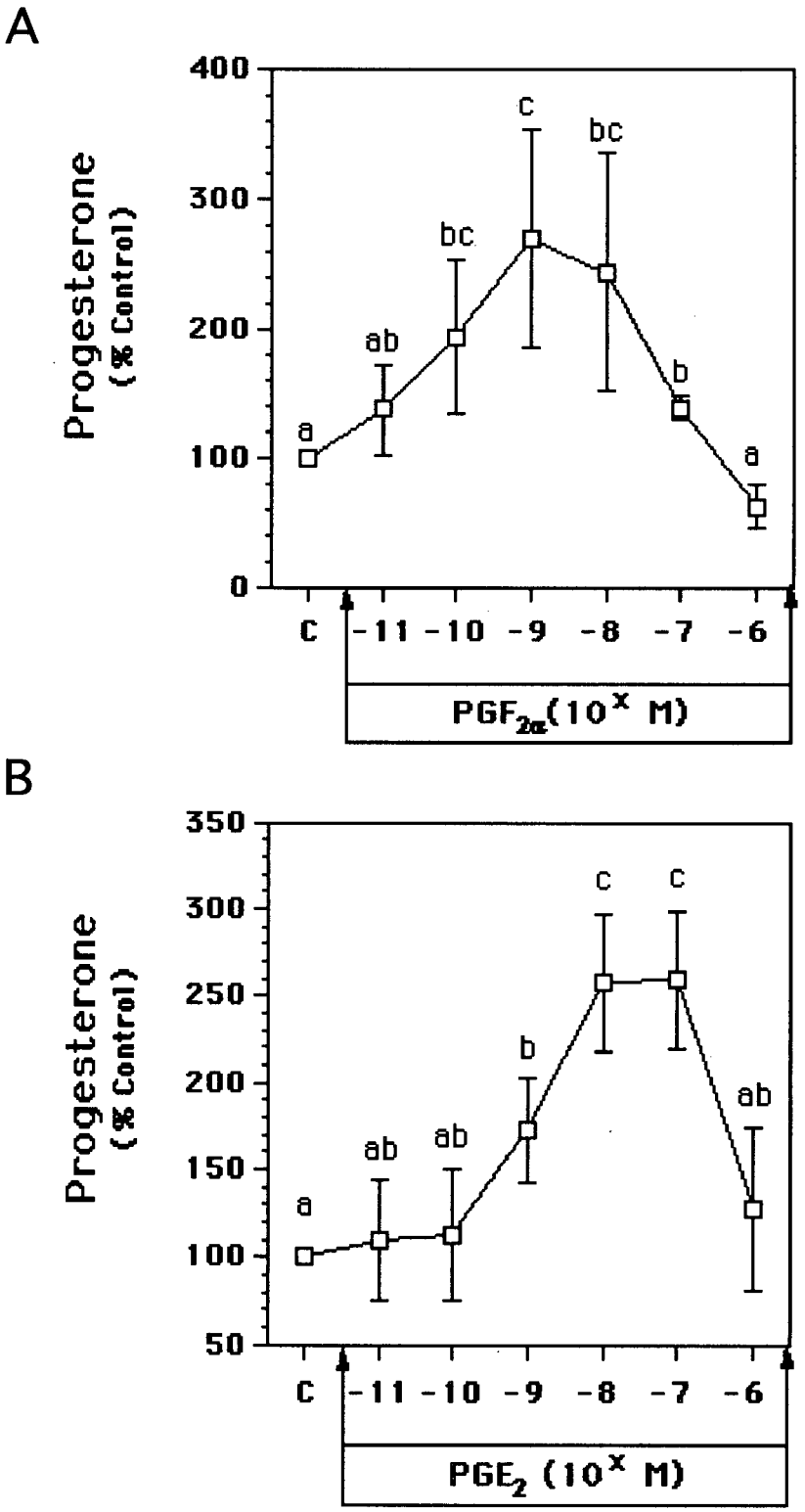


Figure 45. Prostaglandin $F_{2\alpha}$ (**A**; $PGF_{2\alpha}$) and prostaglandin E_2 (**B**; PGE_2) concentration response curves (10^{-11} to 10^{-6} M), in eight day pre-cultured human granulosa-luteal cells. Progesterone was significantly stimulated (over 24 h) in a bell curve-like manner by $PGF_{2\alpha}$ with maximal stimulation at 1 nM ($a \neq b \neq c$, $p<0.05$ by ANOVA). A similar response to PGE_2 was seen although the bell curve was shifted right. Maximal PGE_2-mediated stimulation of progesterone production was seen at 10 to 100 nM. These data represent a two dimensional slice of the data presented in Figure 43.

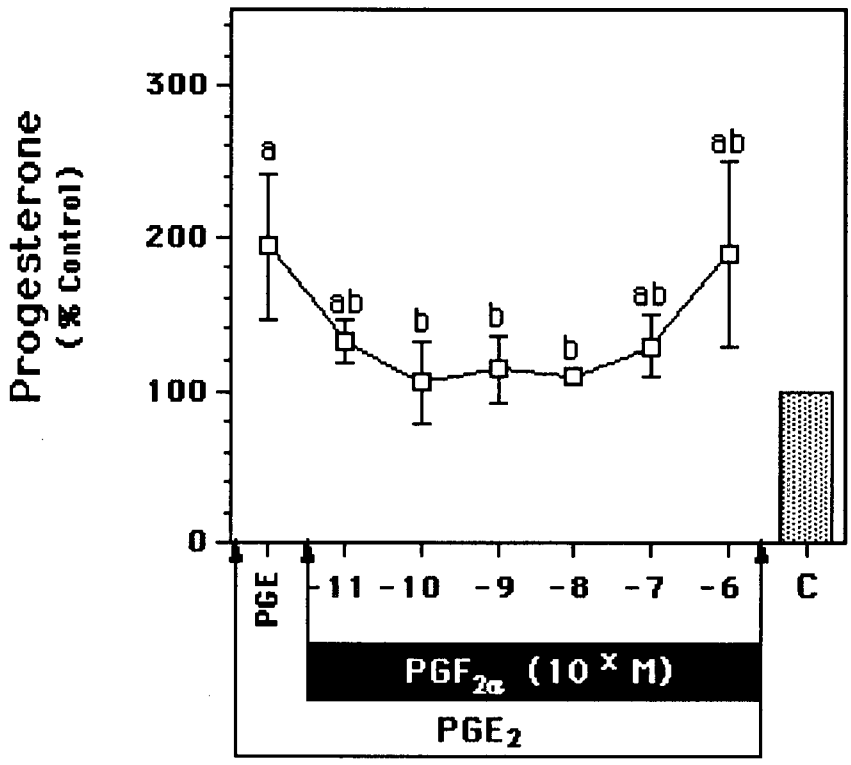


Figure 46. The effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-11} to 10^{-6} M), in the presence of prostaglandin E_2 (PGE_2; 10^{-7} M). Progesterone production (over 24 h) was significantly inhibited ($a \neq b$, $p<0.05$ by ANOVA) in an inverse bell curve-like manner by $PGF_{2\alpha}$ (10^{-10} to 10^{-8} M), in eight day pre-cultured human granulosa-luteal cells. These data represent a two dimensional slice of the data presented in Figure 43.

D. Signal Transduction of $PGF_{2\alpha}$-Mediated Luteolysis

Effects of $PGF_{2\alpha}$ on hCG-Stimulated Steroidogenesis

Progesterone (Fig. 47A) and estradiol (Fig. 47B) production were stimulated by hCG (1 IU/ml; $p<0.05$ control vs. hCG) in eight-day cultured human granulosa-luteal cells, although hCG-stimulated progesterone and estradiol production were both attenuated in the presence of $PGF_{2\alpha}$ (10^{-6} M; $p>0.05$ control vs hCG plus $PGF_{2\alpha}$).

Effects of $PGF_{2\alpha}$ on Isoproterenol Stimulated Progesterone Production

The ß-adrenergic agonist isoproterenol (10^{-5} M) was capable of stimulating progesterone production from eight-day cultured human granulosa-luteal cells (Fig. 48A; $p<0.05$; control vs isoproterenol). As with hCG, isoproterenol-stimulated progesterone production was blocked by the addition of $PGF_{2\alpha}$ (10^{-6} M) to culture media ($p>0.05$; control vs. isoproterenol/$PGF_{2\alpha}$; $p<0.05$ isoproterenol vs isoproterenol/$PGF_{2\alpha}$). Isoproterenol also stimulated estradiol production from human granulosa-luteal cells (Fig. 48B; $p<0.05$, control vs. isoproterenol). The ability of $PGF_{2\alpha}$ to inhibit isoproterenol-stimulated estradiol production was not examined in these studies. Please note that $PGF_{2\alpha}$ transiently stimulated progesterone production (compare Fig. 47A with Fig. 48A). The cause of this transience is under study in another project.

Effects of PTX on Anti-gonadotrophic Actions of $PGF_{2\alpha}$

Treatment of eight-day cultured human granulosa luteal cells with hCG (1 IU/ml) significantly stimulated progesterone production (Fig. 47A and Fig. 49A; $p<0.05$ versus control). Furthermore, this stimulation was inhibited by co-treatment with $PGF_{2\alpha}$ (10^{-6} M; $p<0.05$ versus hCG and $p>0.05$ versus control). However, in the presence of PTX (50 ng/ml), $PGF_{2\alpha}$-mediated inhibition of hCG-stimulated progesterone production was blocked ($p<0.05$ versus control and $p>0.05$ versus PTX/hCG treated cells). Similar progesterone responses were seen from one-day cultured cells under the same conditions (not shown). Estradiol production from eight-day cultured human granulosa-luteal cells paralleled progesterone responses under the above treatment conditions (Fig. 49B). DNA levels remained unchanged by this treatment regime (Fig. 50) suggesting that steroid responses were not due to altered cell numbers in these experiments.

Effects of $PGF_{2\alpha}$ on CTX Stimulated Steroidogenesis

Cholera toxin (1 μg/ml) significantly stimulated progesterone production from eight-day cultured human granulosa-luteal cells (Fig. 51A; $p<0.05$, CTX versus control). Subsequently, $PGF_{2\alpha}$ (10^{-6} M) was able to block the stimulatory effect of CTX ($p<0.05$, CTX versus CTX plus $PGF_{2\alpha}$; and $p>0.05$ for control versus CTX/$PGF_{2\alpha}$). However, co-treatment with PTX (50 ng/ml) partially reversed this effect. Estradiol production in response to these treatments followed a similar profile (Fig. 51B).

Effects of $PGF_{2\alpha}$ on Forskolin Stimulated Progesterone Production

Forskolin (10^{-5} M) significantly stimulated progesterone production from eight-day cultured human granulosa-luteal cells (Fig. 52A and 52B; $p<0.05$, forskolin vs. control). $PGF_{2\alpha}$ (10^{-6} M) was able to block the stimulatory effect of forskolin ($p<0.05$, forskolin vs. forskolin plus $PGF_{2\alpha}$; $p>0.05$ control vs. forskolin plus $PGF_{2\alpha}$). Please note that $PGF_{2\alpha}$ alternately stimulated ($p<0.05$, control vs. $PGF_{2\alpha}$) or did not stimulate progesterone production on its own (compare Fig. 47A and 48B), although this transience did not alter the inhibitory properties of $PGF_{2\alpha}$.

Effects of $PGF_{2\alpha}$ on cAMP Stimulated Progesterone Production

Db-cAMP was capable of stimulating progesterone production from eight-day cultured human granulosa-luteal cells ($p<0.05$, control vs. Db-cAMP), although in these experiments, Db-cAMP-stimulated progesterone production was not inhibited by $PGF_{2\alpha}$ (Fig. 53).

The Effects of a PKC Inhibitor on $PGF_{2\alpha}$-Mediated Inhibition of hCG-Stimulated Progesterone Production.

Human chorionic gonadotrophin (1 IU/ml) significantly stimulated progesterone production in D_8 cultured human granulosa-luteal cells (Fig. 54; n=4). Moreover, the highly specific protein kinase-C inhibitor bisindolylmaleimide (50 nM) significantly inhibited ($p<0.05$) $PGF_{2\alpha}$-mediated inhibition of hCG-stimulated progesterone production in these cells.

(see p. 132 for relevant discussion)

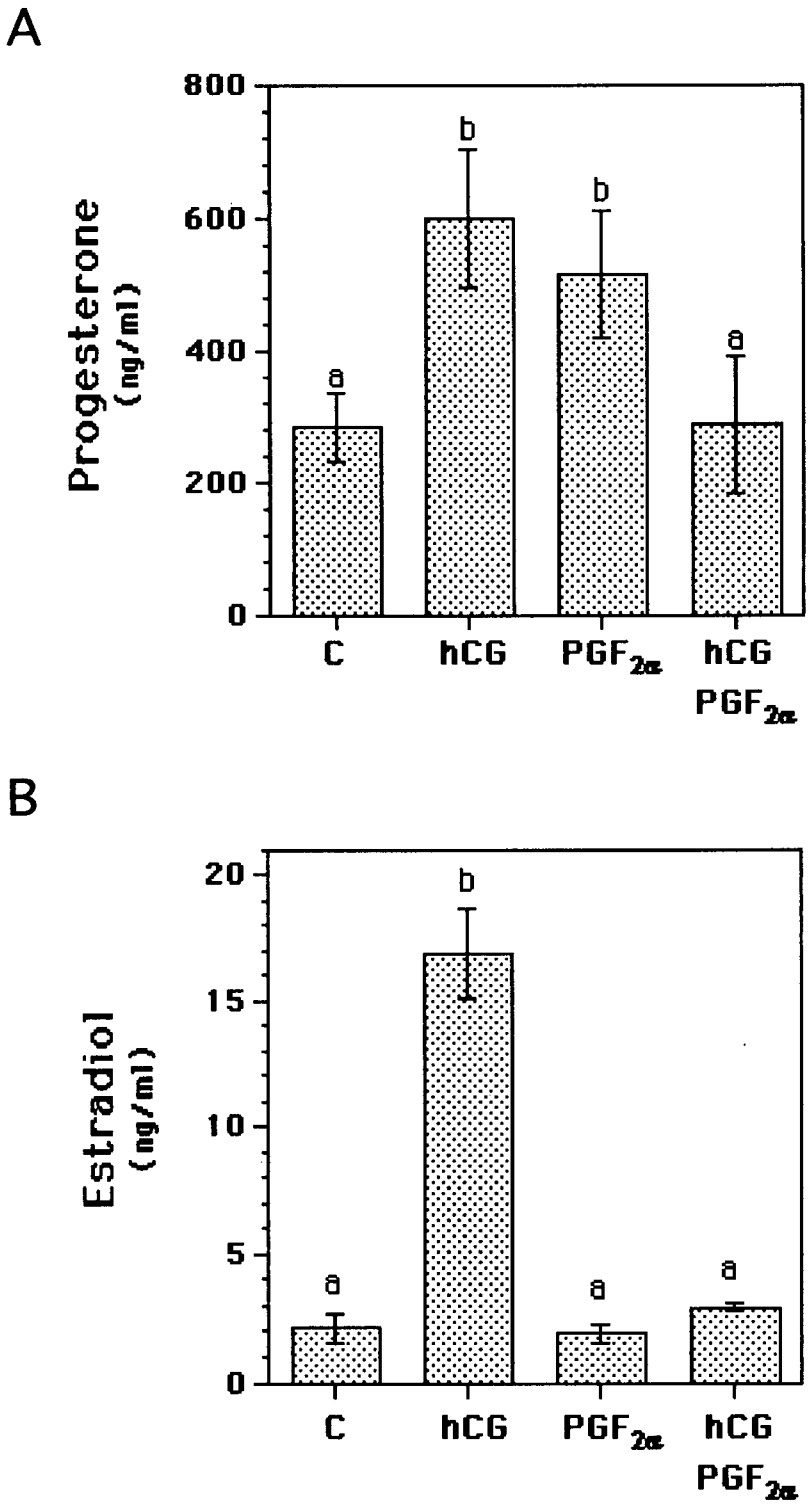


Figure 47. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M)-mediated inhibition of human chorionic gonadotrophin (hCG; 1 IU/ml)-stimulated progesterone (**A**) and estradiol (**B**) production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures ($a \neq b$; $p<0.05$; by ANOVA). Similar results were seen in fourteen separate experiments performed on cells from fourteen other patients.

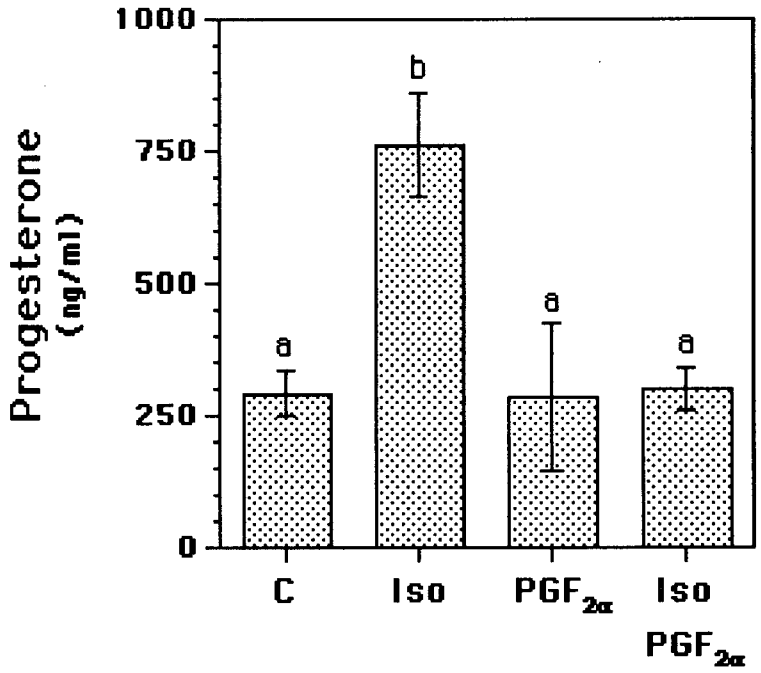


Figure 48. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M)-mediated inhibition of isoproterenol (Iso; 10^{-5} M)-stimulated progesterone production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures ($a \neq b$; $p<0.05$; by ANOVA). Similar results were seen in three separate experiments performed on cells from three other patients.

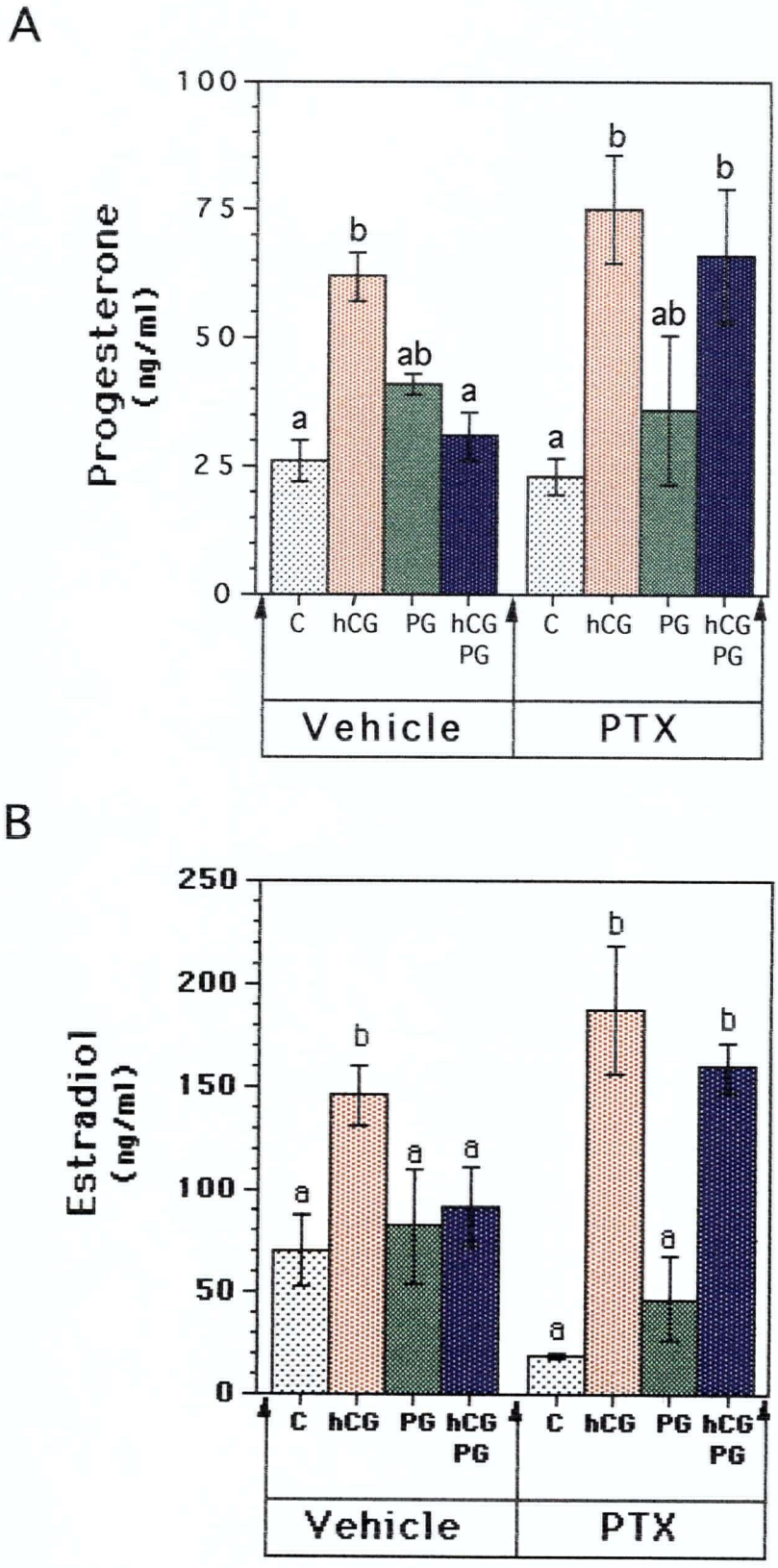


Figure 49. The effects of pertussis toxin (PTX 50 ng/ml) on prostaglandin $F_{2\alpha}$ (PG; 10^{-6} M)-mediated inhibition of human chorionic gonadotrophin (hCG; 1 IU/ml)-stimulated progesterone (**A**) and estradiol (**B**) production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures ($a \neq b$; $p<0.05$; by ANOVA). Similar results were seen in five separate experiments performed on cells from five other patients.

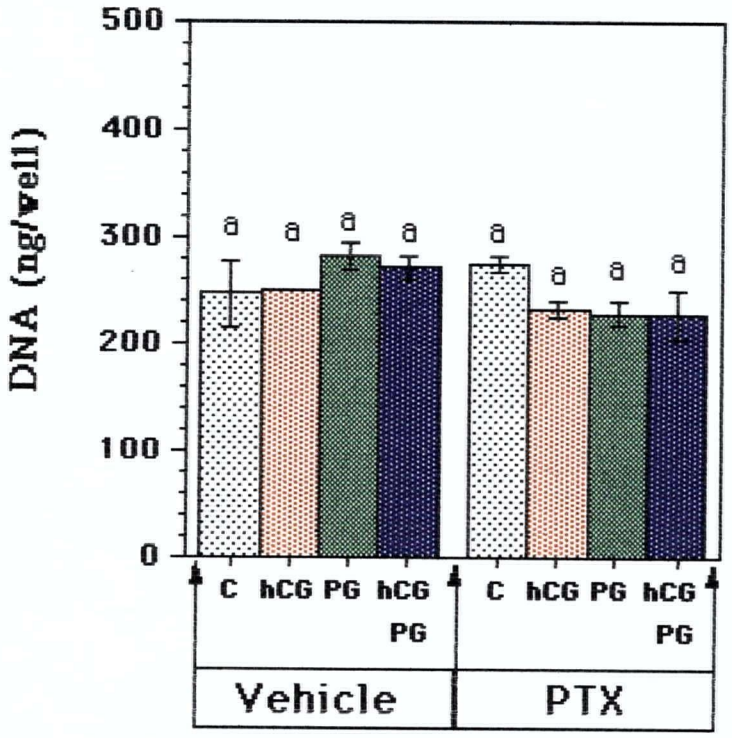


Figure 50. The effects of pertussis toxin (PTX 50 ng/ml), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M) and human chorionic gonadotrophin (hCG; 1 IU/ml) on DNA levels (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures ($p>0.05$). Similar results were seen in two separate experiments performed on cells from two other patients.

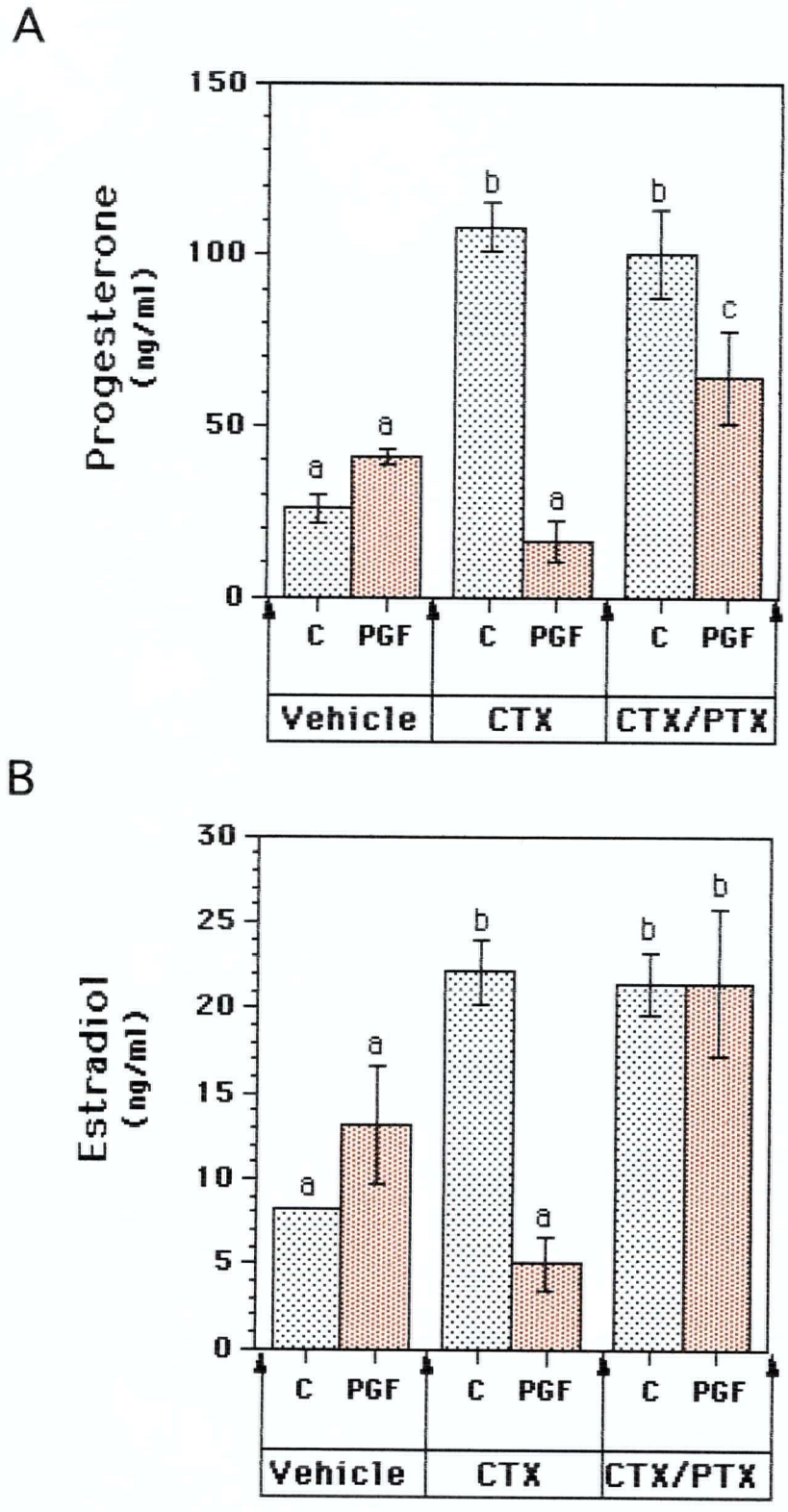


Figure 51. The effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M) on cholera toxin (CTX; 1 μg/ml) and CTX plus pertussis toxin (PTX; 50 ng/ml) stimulated progesterone (**A**) and estradiol (**B**) production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures ($a \neq b$; $p<0.05$; by ANOVA). Similar results were seen in five separate experiments performed on cells from five other patients.

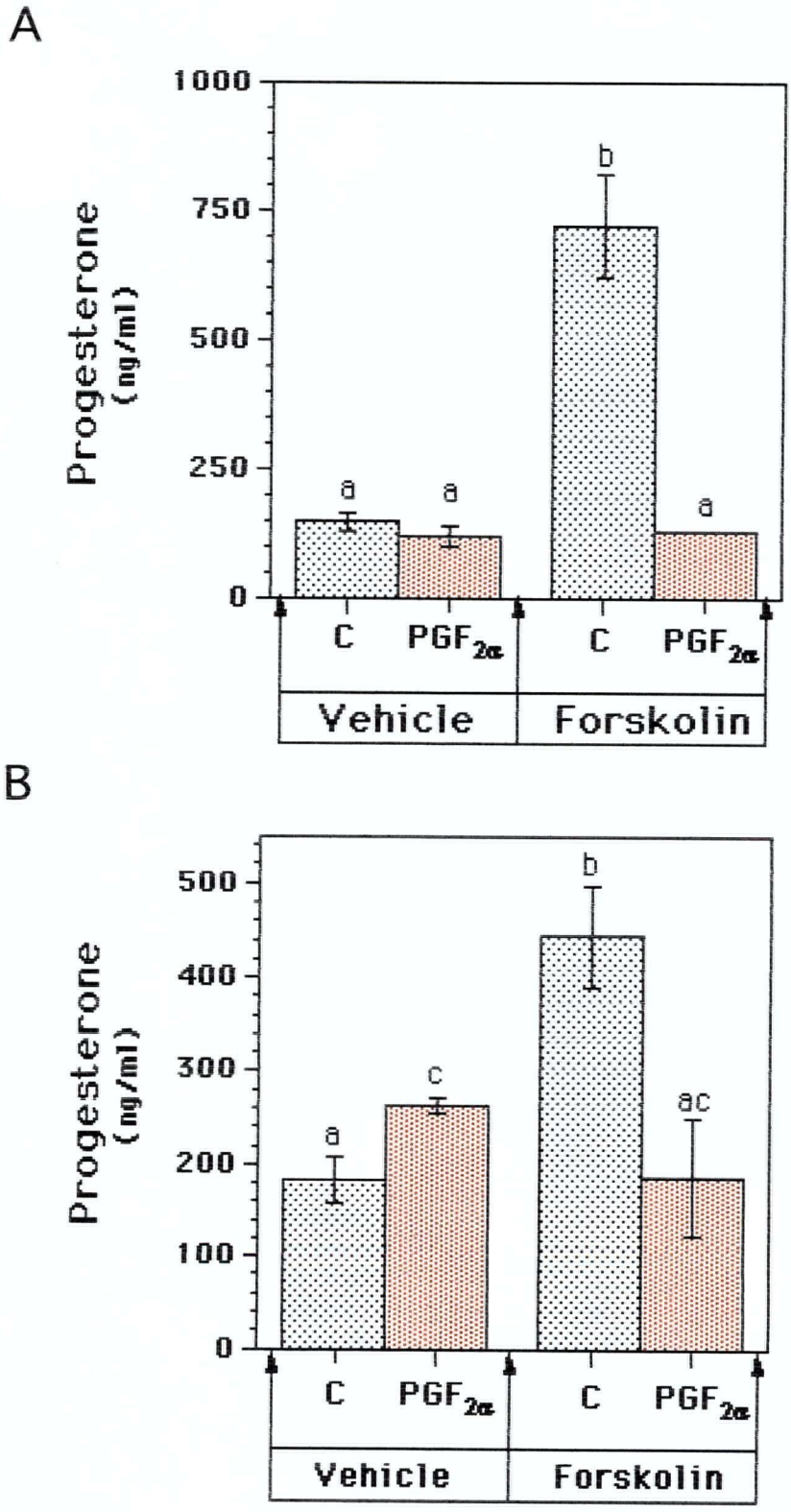


Figure 52. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M)-mediated inhibition of forskolin (10^{-6} M)-stimulated progesterone production (over 24 h), in eight day pre-cultured human granulosa-luteal cells (**A** and **B**). Data represent the mean ± SEM of triplicate measures ($a \neq b \neq c$; $p<0.05$; by ANOVA). Similar results were seen in three separate experiments performed on cells from three other patients. Note: transient stimulatory effect of $PGF_{2\alpha}$ (10^{-6} M, **A** vs **B**)

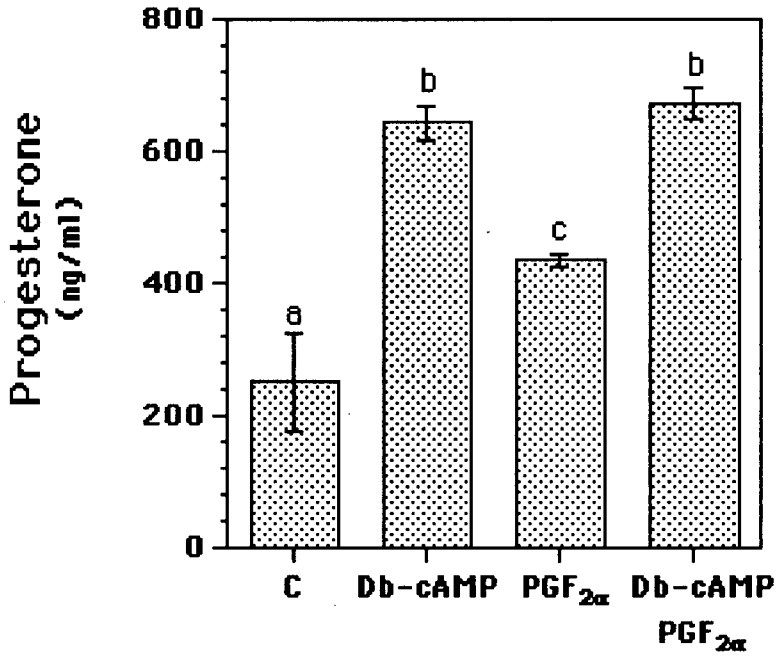


Figure 53. The effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6} M) on dibutryl cAMP (Db-cAMP; 10^{-5} M)-stimulated progesterone production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures ($a \neq b$; $p<0.05$; by ANOVA). Similar results were seen in three separate experiments performed on cells from three other patients. Additionally, similar results seen with experiments utilizing 8-bromo-cAMP, on cells from two other patients.

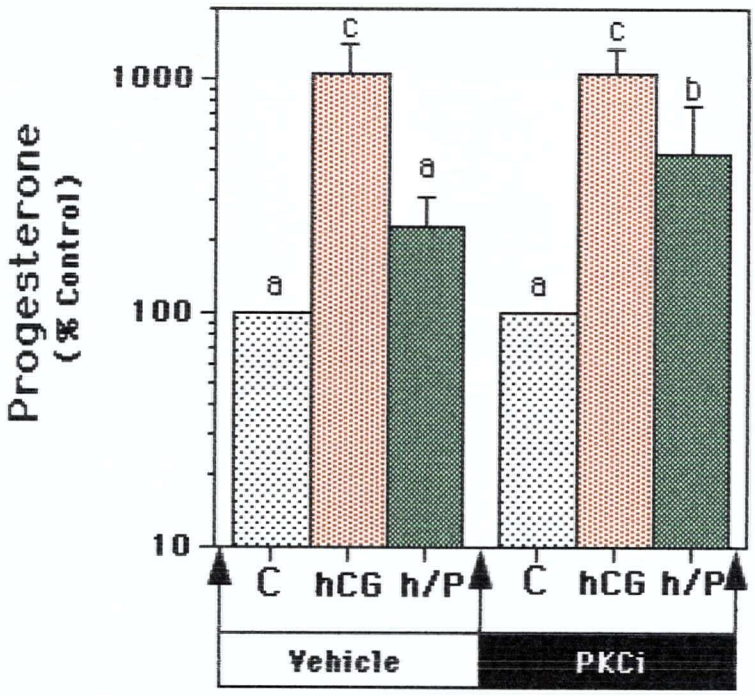


Figure 54. The effects of a protein kinase-C inhibitor (PKCi; bisindolylmaleimide 50 nM) on vehicle (⬚), human chorionic gonadotrophin (hCG; ▨) or hCG plus prostaglandin $F_{2\alpha}$ (h/P; ▩)-mediated inhibition of hCG-stimulated progesterone production (over 24 h; n=4; a≠b≠c, $p<0.05$ by ANOVA), in eight day pre-cultured human granulosa-luteal cells.

E. Effects of hCG and $PGF_{2\alpha}$ on $PGF_{2\alpha}$-R-mRNA

Spectrophotometric Estimation of Known DNA Levels in Solution

Known quantities of DNA were estimated with spectrophotometric analysis in order to validate the spectrophotometer as a tool for approximating DNA and/or RNA levels in samples to be reverse transcribed. Concentrations of DNA between 5 and 5000 ng/ml were sampled. Overall, the spectrophotometric estimation of DNA levels were within 133.8 ± 5.0% of the actual DNA concentration. At high concentrations (1250 - 5000 ng/ml), this estimation improved to 100.8 ± 0.2% of the actual DNA concentration. See Table 7 for a complete listing of the results.

RNA Integrity and Relative Quantity

The integrity and relative quantity of total RNA samples extracted from human GLCs were checked by denaturing (formaldehyde) agarose (1.5%) gel electrophoresis. The presence of 28 and 18 S bands suggested that RNA was intact. Moreover, the apparent consistency of signal strength from one sample to the next suggested that similar efficiency of extraction was obtained for all samples. Data from two different experiments are presented here (Fig. 55 A and B). Similar results were found in other experiments.

PCR Cycle Experiment

Polymerase chain reaction cycle experiments for $PGF_{2\alpha}$-R and ß-Actin cDNA were performed in order determine the optimal number of cycles for a given concentration range and species of cDNA (Fig. 56). At the concentrations of cDNA utilized in these experiments, PCR amplification of product was relatively linear over the range of cycles tested. Amplification of cDNA for and $PGF_{2\alpha}$-R and ß-Actin was performed using 40 and 30 cycles, respectively, based on the results of these experiments.

Amplification of $PGF_{2\alpha}$-R and ß-Actin cDNAs in Human GLCs

Prostaglandin $F_{2\alpha}$-R cDNA was amplified from human GLCs (obtained from 2 different patients) with two different sets of oligonucleotide primers (hPGF+/- and rPGF+/-). Products of the expected size (802 and 720 bp) were amplified by both primers (hPGF+/- and rPGF+/-; Fig. 57A and B). Additionally, oligonucleotide primers for ß-actin cDNA (Act+/-) successfully amplified a product of the expected size (524 bp) from human GLCs obtained from 3 different patients (Fig. 57C).

Confirmation of $PGF_{2\alpha}$-R cDNA in Human Granulosa-Luteal and Placental Cells

Amplification of $PGF_{2\alpha}$-R cDNA using PCR incorporating ^{32}P-dCTP revealed the presence of products in samples obtained from human GLCs from three separate patients and in placental cells from two separate patients (Fig. 58A). However, PCR failed to detect $PGF_{2\alpha}$-R cDNA in human leukocyte cDNA samples from two patients. The photograph of this gel was further validated when lanes from this experiment were cut and counted with a ß-counter. Similar results were demonstrated using this technique.

Regulation of $PGF_{2\alpha}$-R cDNA by hCG and $PGF_{2\alpha}$

One-day cultured human GLCs were incubated with vehicle, hCG (1 IU/ml) or hCG plus $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M). The effects of these treatments on $PGF_{2\alpha}$-R and ß-Actin cDNA levels were examined by RT-PCR (Fig. 59A), densitometry (Fig. 59B) and Southern blot hybridization (Fig. 60A and B).

Briefly, $PGF_{2\alpha}$-R message was down-regulated by hCG. However, $PGF_{2\alpha}$ at low (10^{-11} M) and high (10^{-6} M) concentrations prevented this down-regulation. On the contrary, the middle concentration of $PGF_{2\alpha}$ (10^{-9} M) potentiated hCG-mediated down-regulation of $PGF_{2\alpha}$-R message (Fig. 59A). Densitometric analysis revealed significant inhibition of $PGF_{2\alpha}$-R mRNA levels in cells treated with hCG and hCG plus $PGF_{2\alpha}$ (n=3; $p<0.05$ by ANOVA; 10^{-9} M; Fig. 59B). The housekeeping gene ß-actin was unaffected by any of the above treatments (not shown).

Southern blot hybridization of a semi-quantitative PCR experiment (presented in Figure 59A), with an oligonucleotide probe confirmed the identity of the PCR products (Fig. 60A). Moreover, densitometric analysis of the autoradiogram revealed a pattern of mRNA regulation similar to that found in Figure 59B. In short, hCG inhibited $PGF_{2\alpha}$-R mRNA message in this experiment. Additionally, in presence of hCG, $PGF_{2\alpha}$ (10^{-11} to 10^{-7} M) inhibited $PGF_{2\alpha}$-R mRNA message, potentiating hCG-mediated inhibition at concentrations of (10^{-10} to 10^{-8} M, $PGF_{2\alpha}$).

(see p. 142 for relevant discussion)

Table 7. Spectrophotometer estimation of known DNA levels in solution.

DNA by Weight * (ng/ml)	**Spectrophotometer Estimation (% Actual Conc.± SEM)**
5 to 5000	**133.8 ± 5.0**
5 to 40	183.6 ± 12.0
80 to 625	111.1 ± 1.6
1250 to 5000	100.8 ± 0.2

* Salmon sperm DNA serially diluted in 2x dilutions) from 5000 to 5 ng/ml.

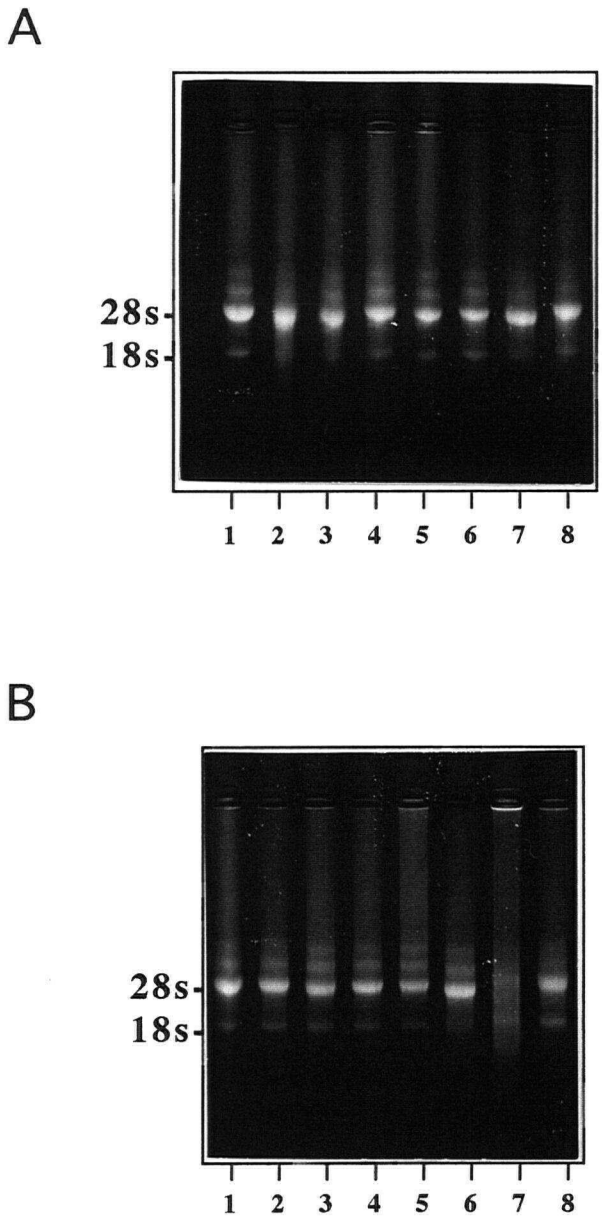


Figure 55. RNA integrity gel. Agarose (1.5 %) and formaldehyde denaturing gel of RNA samples (1 μg/sample) extracted from one day pre-cultured human granulosa-luteal cells. The presence of 28 and 18 S bands suggests that RNA integrity was good. RNA from two experiments is presented here (**A** and **B**).

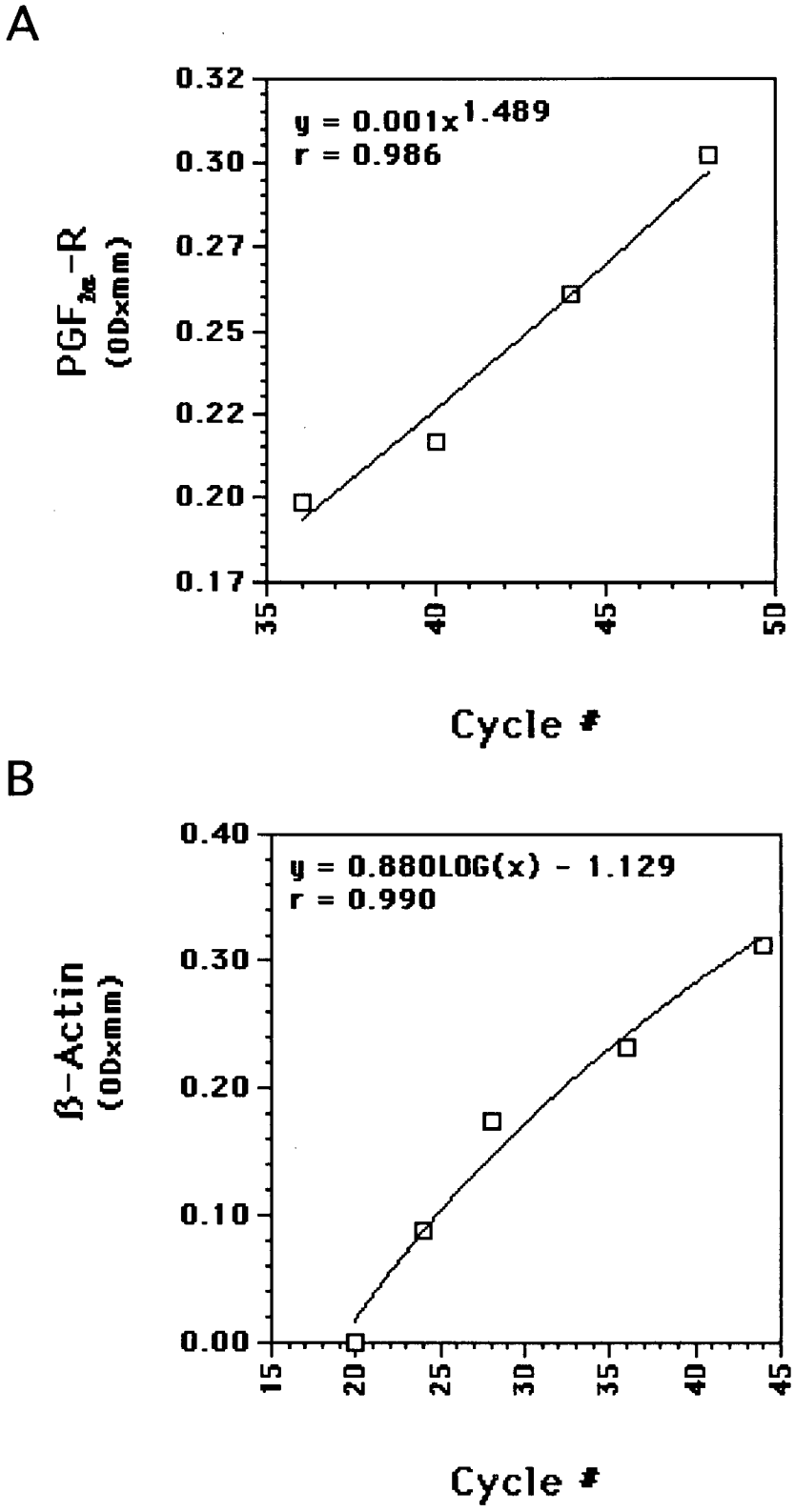


Figure 56. Polymerase chain reaction (PCR) cycle experiments for prostaglandin $F_{2\alpha}$-receptor (**A**; $PGF_{2\alpha}$-R) and ß-Actin (**B**) complementary DNA (cDNA). At the concentrations of cDNA utilized in these experiments PCR amplification of product was relatively linear over the range of cycles tested. Based on these experiments PCR amplification of cDNA for and $PGF_{2\alpha}$-R and ß-Actin was performed using 40 and 30 cycles, respectively.

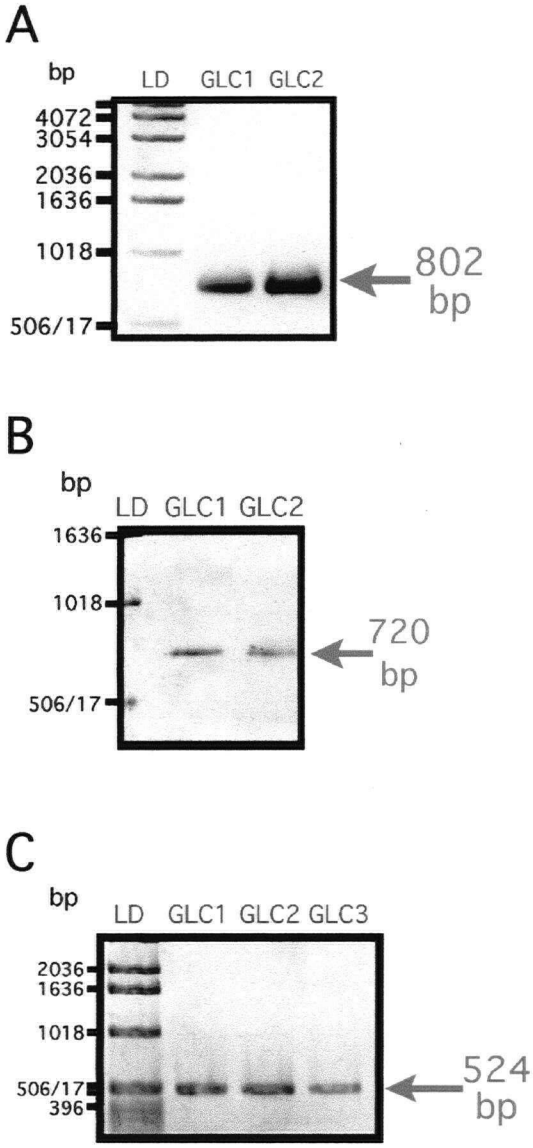


Figure 57. Polymerase chain reaction amplification of $PGF_{2\alpha}$-R and ß-actin cDNA. Two different sets of oligonucleotide primers were utilized to amplify prostaglandin $F_{2\alpha}$-receptor cDNA, and one set was utilized to amplify ß-actin cDNA, from one day pre-cultured human granulosa-luteal cells. These primers were hPGF+ and hPGF- (**A**), rPGF+ and rPGF+ (**B**) and Act+ and Act- (**C**). All three sets of oligonucleotides were able to amplify products of the predicted size, from human granulosa-luteal cells (GLC) from up to three different patients.

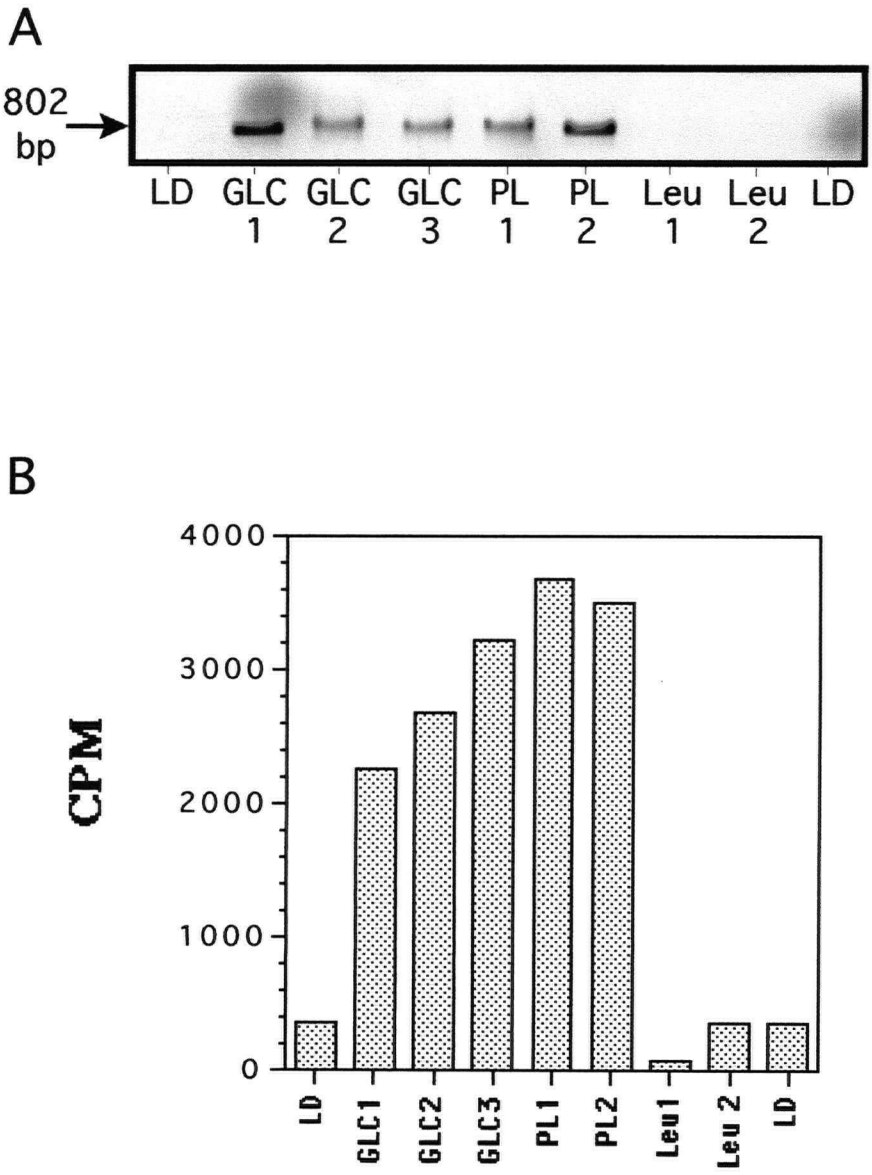


Figure 58. Polymerase chain reaction amplification of $PGF_{2\alpha}$-receptor cDNA from human granulosa-luteal cells, placenta and leukocytes. **A.** Amplification of the prostaglandin $F_{2\alpha}$-receptor cDNA with the oligonucleotides hPGF+ and hPGF-, in the presence of ^{32}P-dCTP. Polymerase chain reaction products of the predicted size were amplified from three different human granulosa-luteal cell (GLC1, 2 and 3; uncultured samples) and two different human placenta (PL1 and 2; uncultured samples) cDNA samples. Conversely, gel lanes loaded with molecular weight ladder (LD) or two different Leu cDNA samples (uncultured samples), did not show visible amplification of product. **B.** Gel bands (from **A**) separated and counted with a ß-counter.

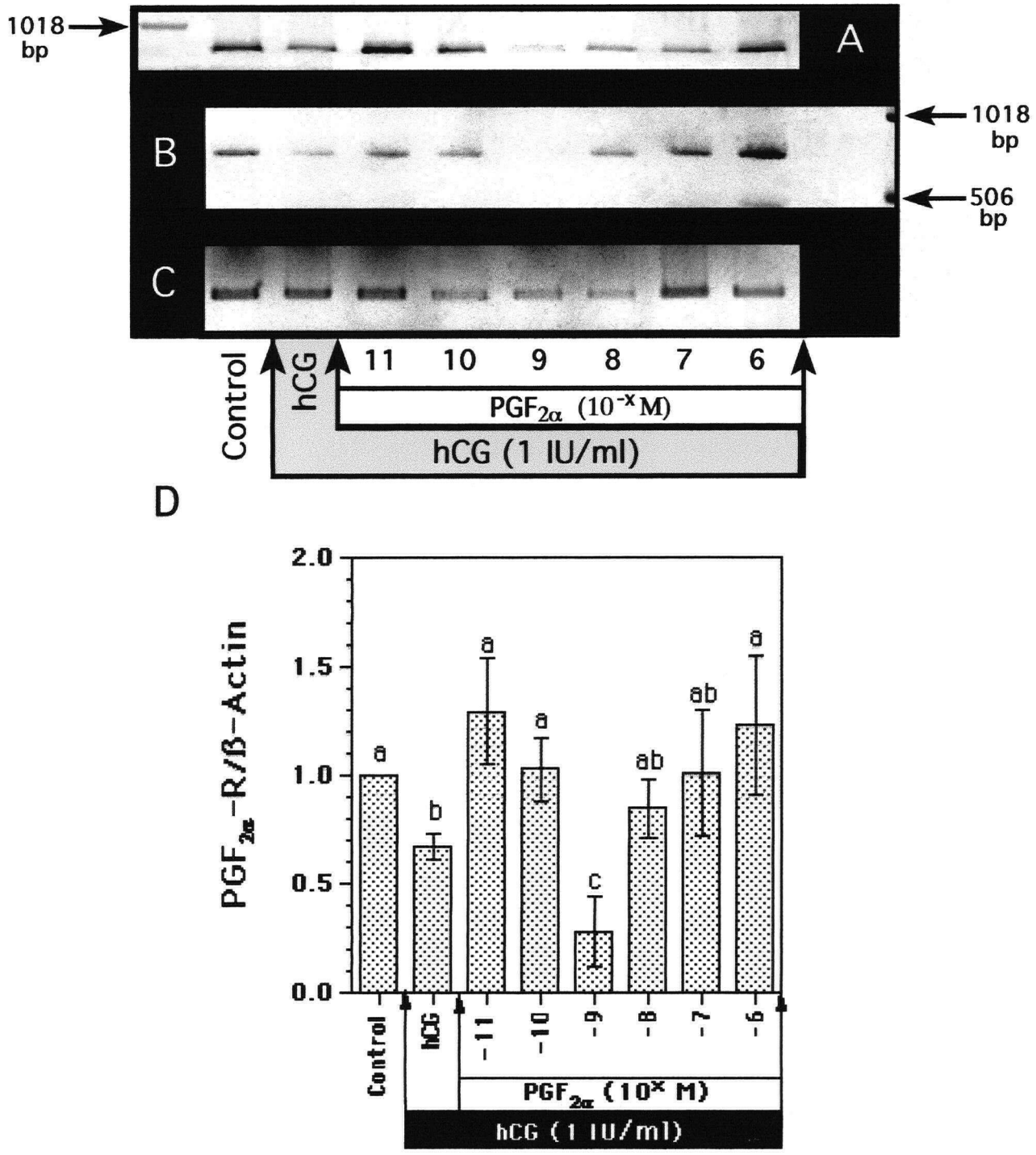


Figure 59. The effects of vehicle, human chorionic gonadotrophin (hCG) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on $PGF_{2\alpha}$-receptor ($PGF_{2\alpha}$-R) mRNA levels (over 24 h), in one day pre-cultured human granulosa-luteal cells from three separate patients (**A**, **B** and **C**). Following the treatment period cells were extracted for RNA, which was reverse transcribed (RT) to cDNA and subjected to semi-quantitative polymerase chain reaction (PCR). $PGF_{2\alpha}$-R message was down regulated by hCG, however, $PGF_{2\alpha}$ at low (10^{-11} M) and high (10^{-6} M) concentrations prevented this down-regulation. On the contrary, the middle concentration of $PGF_{2\alpha}$ (10^{-9} M) potentiated hCG-mediated down-regulation of $PGF_{2\alpha}$-R message. The house keeping gene ß-actin was uneffected by any of the above treatments (not shown). Photographs were subjected to densitometric analysis, and normalized to ß-actin responses and averaged (**D**). Significant inhibition of $PGF_{2\alpha}$-R mRNA levels was seen in cells treated with hCG and hCG plus $PGF_{2\alpha}$ ($a \neq b \neq c$. $p<0.05$ by ANOVA; 10^{-9} M).

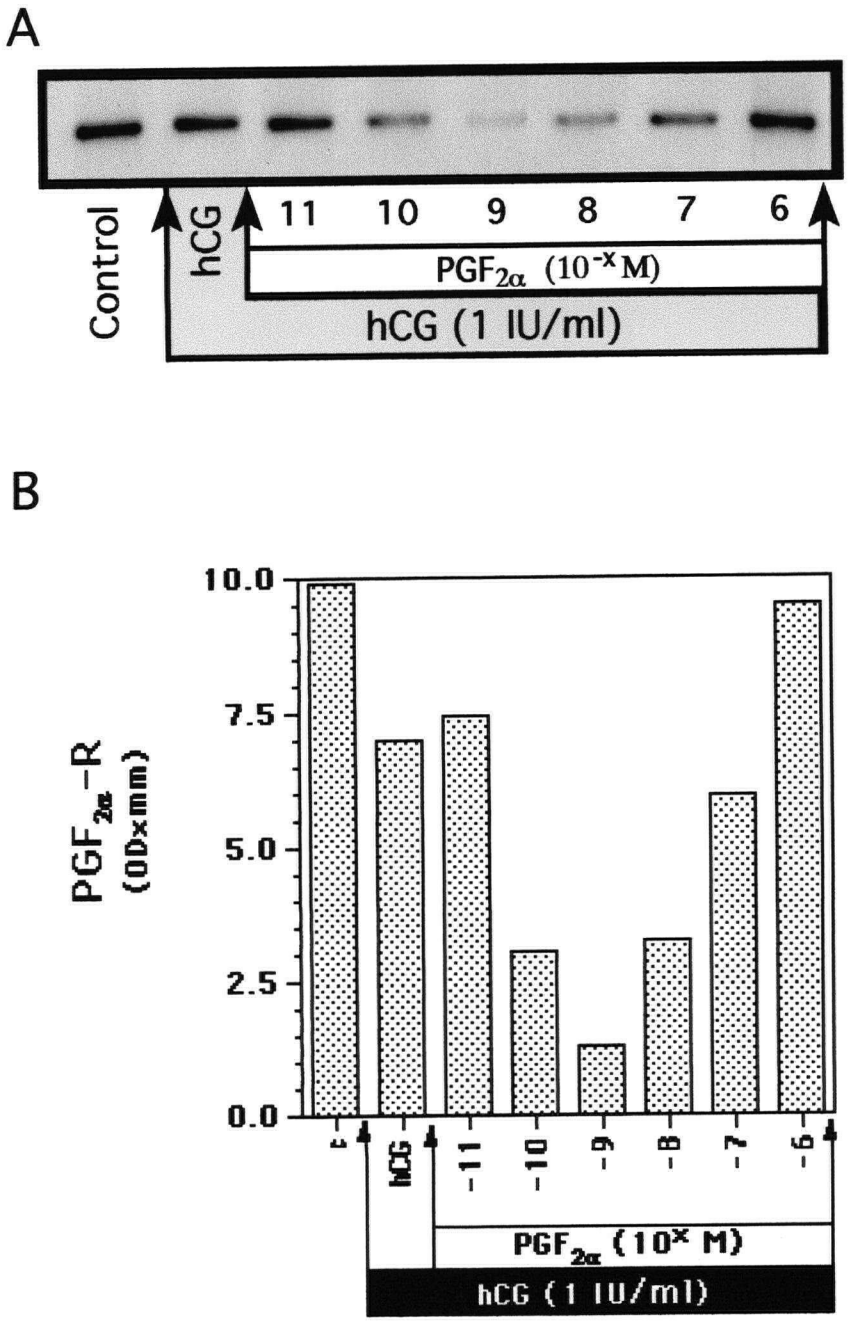


Figure 60. A. Southern blot hybridization of a semi-quantitative PCR experiment, with an oligonucleotide probe for $PGF_{2\alpha}$-R, in cells from one day pre-cultured human granulosa-luteal cells. These data confirm the identity of the PCR products presented in (Fig. 59 A). **B.** Densitometric analysis of the autoradiogram presented in A. Human chorionic gonadotrophin (hCG) inhibited $PGF_{2\alpha}$-R mRNA message in this experiment. Moreover, in the presence of hCG, $PGF_{2\alpha}$ (10^{-11} to 10^{-7} M) inhibited $PGF_{2\alpha}$-R mRNA message, potentiating hCG-mediated inhibition at concentrations of (10^{-10} to 10^{-8} M, $PGF_{2\alpha}$).

VII – DISCUSSION

Caveats of the Human Granulosa-Luteal Cell Model

Variability in Basal Steroidogenesis in the Human GLC Model

Granulosa-luteal cells obtained during oocyte collection from superovulated *in vitro* fertilization patients are a very difficult model to work with as they exhibit a high degree of variability in their basal levels of steroidogenesis (Fig. 13-16).

In part, these differences can be accounted for by varying proportions of cells obtained from different-sized follicles (Fig. 25) since the number and maturity of follicles punctured varies greatly with patients. Additionally, the possiblity of contaminant epithelial or immune cell involvement in the $PGF_{2\alpha}$-mediated responses of luteal cells may further complicate matters, should this exist. Epithelial cells are reported to permit or even enhance $PGF_{2\alpha}$-mediated responses in luteal cells in the placenta [Alecozay et al., 1991]. Thus, differences in follicle puncture, cell collection and purification can all contribute to variability in steroidogenic responses.

Other potential sources of variability include the following: 1) the population of patients from which cells are collected have a much higher probability of infertility than the general population; 2) patient responses to super-ovulation are highly variable, suggesting important biological differences; and 3) the differences in time between follicle puncture and cell plating.

Throughout these experiments cells were plated at a density of 10^3 to 10^4 cells/well. Within this range of cell densities, no significant density-dependent difference was seen in the steroidogenic responses to hCG or hCG plus $PGF_{2\alpha}$ between patients (differences within a patient were not examined). Moreover, basal steroidogenesis did not change in response to cell density. However, in a report by Bari-Ami and Gitay-Goren [1993], basal steroidogenesis showed cell density and culture-time dependent changes in progesterone and estradiol production such that increases in cell density from $2.5x10^3$ to $1x10^4$ cells/well could increase progesterone production 1.3-fold. A further increase in cell density from $1x10^4$ to $8x10^4$ cells/well could decrease progesterone production 3.7-fold. They also reported density and culture time-dependent changes in estradiol production. Culture condition, cell manipulation or other factors may account for the discrepancies in these two studies.

It should be noted, however, that in most cases, although the basal levels of steroidogenesis varied greatly, the relative responses to stimuli were quite similar when comparing responses from different patients (see Fig. 17). Thus, although the problem of variability exists, standardization of data can still render this model useful for determining relative trends and mechanisms of action. Data standardization can be achieved by either experimental or statistical means.

Experimental methods normally include taking the ratio of the data from one physiological parameter over some other form of data. For example: peptide secretion from endocrine cells is often represented over the total amount of stored peptide. Taking the ratio of a response over DNA levels or cellular protein content is also common. In this model, experimental methods of standardization are not satisfactory, as response per cell or DNA level is too variable. Therefore, a statisitical method of standardization is required.

Statistical standardization methods include converting data to a percentage of the control level, log transformations or conversion to standard mean scores [Lewis 1984]. All of these methods have advantages and disadvantages. Log transformations are not completely satisfactory with respect to this model as basal levels can vary many-fold. Thus, these studies utilized percentage of control and standard mean score transformations.

Although the human GLC model is quite variable in the basal levels of steroidogenesis, the relative responses to stimuli are similar in cells from different patients. Thus, this model can be effectively utilized to determine relative responses to stimuli and the mechanisms of these responses. Moreover, this model provides the only source of human granulosa-luteal cells ethically available in high enough quantity to study effectively.

Cell Numbers and Low Level RNA Expression

Although human GLCs are readily available in high enough quantity for morphological or pharmacological study, the numbers of cells obtained are barely adequate for molecular biological techniques. This is especially true when examining genes which are expressed at low levels. Reverse transcription-PCR allowed for semi-quantitative examination of genes of low level expression, due to the amplification obtained through PCR. Even with this amplification $PGF_{2\alpha}$-R mRNA was difficult to detect. Total RNA levels extracted from cells were normally

between 1 to 2 μg per sample, a level too low to be useful for Northern blot hybridization. Thus, although Northern blot analysis would have been an easier and more direct quantification method, the levels of $PGF_{2\alpha}$-R mRNA did not permit this procedure.

Rather than not study the regulation of $PGF_{2\alpha}$-R mRNA, RT-PCR was utilized to provide some useful insights that would not otherwise be possible.

A Question of Physiological Concentration?

What is a physiological concentration? This is a difficult question to answer. In these studies, concentrations of $PGF_{2\alpha}$, PGE_2 and GnRH ranged from pM to μM levels. This range of concentrations was utilized because *in vivo* data in the human is unobtainable. Moreover, the concentrations which a receptor 'sees' are virtually impossible to determine. Tight junctions, secretion patterns, local degradation, binding proteins, receptor affinity and other factors can greatly alter the effective concentration of a hormone at its site of action.

The female reproductive system is not a homeostatic system, it is a cycling system. Herein lies its appeal to many scientists. In a cycling system, one physiological concentration cannot be assigned to most hormones. For example, progesterone and estradiol concentrations vary greatly throughout the menstrual cycle. Which concentration is physiological? At any given point in time, the physiological concentration of a hormone may change. Additionally, functions can be attributed to a hormone's absence as well as its presence. Finally, as the affinty of the $PGF_{2\alpha}$-R is quite high (Kd of 1 nM), it is reasonable to expect that this hormone may have physiological effects at concentrations which are several-fold lower or higher than this Kd. This turned out to be the case.

Summary

Notwithstanding these limitations, it must be noted that this model is a valuable one. There are very few human tissues which are so obtainable for scientific examination. The author believes that it is a scientist's responsibility to work around the limitations of such a valuable model and learn as much as one can from it.

Morphology of Human Granulosa-Luteal Cells in Culture

The morphological characteristics of human GLCs change dramatically with culture time. Moreover, these changes correspond well with those reported for cells undergoing luteinization. Granulosa cells exposed to luteotrophins change from their characteristic polygonal shape to a round one, occasionally projecting finger-like processes which may attach to adjacent cells [Soto et al., 1986]. These characteristics are very similar to those of freshly cultured and one-day cultured human GLC, as seen in these studies. As cells luteinize, they are reported to form tighter associations [Ratamales et al., 1994], increased vacuolation and blebbing [Quirk et al., 1995]. All of these reported characteristics were seen in eight-day and twelve-day cultures of human GLCs, suggesting that these cells are luteinizing in culture. This notion is certainly supported by the functional differentiation seen with culture time.

An increase in luteal cell blebbing, characteristic of cells undergoing apoptosis, has also been reported in human granulosa-luteal cells [Quirk et al., 1995]. Interestingly, in these studies it was found that an increase in cell irregularity and blebbing was seen as culture time progressed. Moreover, cells disrupted by 16 to 18 days of culture appeared to have undergone apoptosis. This disruption was associated with a loss in functional response.

Similar morphological characteristics have been reported in other mammals including porcine, bovine, feline and rat models [Gregoraszczuk and Krzysztofowicz 1989; Roth et al., 1995; Chegini et al., 1984; Fields et al., 1992; Meidan et al., 1990; Fields et al., 1985; Yuh et al., 1986; Nelson et al., 1992]. Luteal cell vacuolation, blebbing of various sizes, ruffles and lipid vacuoles are also reported in these species. In the rat, differences between small and large luteal cells are also reported, such that small luteal cells appear stellate while large luteal cells do not flatten out completely (probably due to large lipid droplets) [Nelson et al., 1992]. In one-day human GLCs, two cell populations appeared to be present which corresponded remarkably well with those reported in the rat.

In summary, human GLCs appeared to undergo morphological luteinization and possibly apoptosis with culture time. These results support the human granulosa-luteal cell as a good model for the study of luteinization.

A Effects of $PGF_{2\alpha}$ on Human Granulosa-Luteal Cells in the Absence and Presence of hCG

Wide ranging concentration-response studies (1 pM to 1μM $PGF_{2\alpha}$ or GnRH) were performed in both the presence and absence of hCG, in short, medium and long term cultures of human GLCs. The importance of these parameters was highlighted by the concentration- and culture time-dependent differential responses to $PGF_{2\alpha}$ under these conditions. Profoundly different progesterone and estradiol production in response to $PGF_{2\alpha}$ was seen when comparing D_1, D_8 and $D_{12\text{-}14}$ cultured human GLCs. In the case of GnRH, differences were observed from D_1 to D_8 cells. These findings emphasize the importance of maintaining awareness of culture time in experiments using highly differentiated GLCs.

Further, the basal effects of $PGF_{2\alpha}$ suggest that superovulation-derived human GLCs continue to undergo luteinization *in vitro*, as they paralleled previous results examining early- and mid-luteal phase cells [Khan et al., 1989]. The effects of $PGF_{2\alpha}$ on progesterone production in GLCs differed with culture time. Prostaglandin $F_{2\alpha}$ inhibited in day D_1, but stimulated progesterone production in $D_{12\text{-}14}$ cultured GLCs. The cells were found to be less defined in their responses to $PGF_{2\alpha}$ in D_8, as they appeared to be in a state of transition between their inhibitory and stimulatory responses. Early-luteal and D_1 GLCs both demonstrated inhibition, while mid-luteal and D_8 GLCs demonstrated stimulation of basal-progesterone production in response to $PGF_{2\alpha}$ [Khan et al., 1989]. This further supports the suitability of IVF derived cells as a model to study human ovarian cell function.

Thus, $PGF_{2\alpha}$ was capable of either inhibiting or stimulating progesterone production depending on concentration and culture conditions.

Follicle Size

Differential responses to hCG and $PGF_{2\alpha}$ based on follicle size were seen in D_1 GLCs. When the results from four separate experiments were pooled, it became clear that the magnitude of the hCG-induced steroidogenic response was reduced in small versus large follicles. The response was significant in large follicles, but not in small follicles. Previous studies have demonstrated that the number of hCG receptors increases with follicle size [Kammerman and

Ross, 1975; May and Schomberg, 1984; Hillier et al., 1980]. These results suggest that there could be differential steroidogenic responses in cells from different patients, due to differing proportions of small and large follicles. This hypothesis is supported by a previous report which found that follicles yielding mature cumulus-oocyte complexes (COC) represent a non-homogenous population in which GLCs from follicles yielding type A-B COC (cumulus cells aggregated into clumps) are less luteinized than GLCs from follicles yielding type C-D COCs (cumulus cells homogeneously spread out) [Gitay-Goren et al., 1990].

Concentration and Culture Time Dependent Responses

The concentration range of $PGF_{2\alpha}$ and GnRH used in these studies resulted in bell curve like inhibition of hCG-stimulated progesterone and estradiol production (see Fig. 26, 28, 30–31). This bimodal nature is not unusual for prostaglandin actions [Cohen and Rimon, 1992; Sano and Shichi, 1993; Puschel et al., 1993; Hargrove et al., 1975]. For example, in the rat and rabbit testicular tissues, bimodal responses to PGE_2 and PGE_1 have been reported, respectively [Cohen and Rimon, 1992; Hargrove et al., 1975]. Rat epididymal adipocytes displayed a PGE_2-mediated inhibition and stimulation of cAMP at concentrations of 10 mM and >10 mM, respectively. In the rabbit, testicular contractions were stimulated and inhibited by PGE_1 at concentrations of 1–10 nM and 100 nM, respectively. Non-reproductive tissues also have been shown to exhibit bimodal responses to prostaglandins [Sano and Shichi, 1993; Puschel et al., 1993]. In rat hepatocytes, PGE_2 can act in a glycogenolytic and in a antiglycogenolytic fashion at concentrations of 10 μM and 1 nM, respectively. It is reported that these glycogenolytic and antiglycogenolygic actions are likely mediated through the inositol triphosphate and cAMP pathways, respectively. The potential for multiple G-protein coupling to $PGF_{2\alpha}$ receptors is also present as seen in the gonadotroph [Hawes et al., 1993; Barnes and Conn, 1993]. This possiblity is supported by the identification of four different G-protein alpha subunits in the human GLC, including $G_{\alpha s}$, $G_{\alpha i3}$, $G_{\alpha i1,2}$ and $G_{\alpha q,11}$ [Lopez et al., 1995]. Similarily, multiple-receptors have been suggested as an explanation for bimodal prostaglandin responses in the porcine ciliary epithelium [Sano and Shichi, 1993].

In the presence of hCG, culture time (presumably luteinization)-dependent alterations in the steroidogenic responses to $PGF_{2\alpha}$ and GnRH have also been observed. The general trend of the concentration-dependent response to $PGF_{2\alpha}$ or GnRH was retained with culture time although it shifted in an upward (stimulatory) fashion, retaining its anti-gonadotrophic effects only at the highest concentration tested (1 μM), in D_8 GLCs. Potentiation (1.5- to 3-fold) of hCG-stimulated progesterone production was seen D_8 GLCs cultured in the presence of $PGF_{2\alpha}$ or GnRH (at 1 nM; Fig. 3B and 6B). The ability of $PGF_{2\alpha}$ to potentiate hCG-stimulated progesterone production in D_8 (presumably mid-luteal like) cells, may have implications for early pregnancy. Further support for this idea resides in the literature, as hCG pretreatment has been shown to prevent the anti-gonadotrophic actions of $PGF_{2\alpha}$ [Michael and Webley, 1991b]. It has been suggested that hCG produced by the new conceptus may prevent corpus luteum regression by this mechanism. Furthermore, $PGF_{2\alpha}$ concentrations in the human luteal cell are at their highest levels in mid-luteal phase, the time when it would be least appropriate to undergo luteolysis. This potentiation may have an important biological function at this stage, and may involve the interaction of $PGF_{2\alpha}$ and other ovarian hormones such as PGE_2. Prostaglandin E_2 is at its highest concentrations during the mid-luteal phase [Patwardhan and Lanthier, 1980 and 1985] and is reported to have antagonistic actions against $PGF_{2\alpha}$-mediated luteolysis. Perhaps PGE_2-mediated antagonism of $PGF_{2\alpha}$-mediated luteolysis enhances the luteotrophic responsiveness of these cells.

Summary

Prostaglandin $F_{2\alpha}$ and GnRH appear to be bimodal regulators of steroidogenesis in the human ovarian cell. In addition to the antisteroidogenic abilities of $PGF_{2\alpha}$ and GnRH (10^{-6} M; D_1 and D_8), these results suggest that $PGF_{2\alpha}$ (10^{-9} M; D_8 and $D_{12\text{-}14}$) and GnRH (10^{-9} M; D_8) may play a role in the maintenance of the corpus luteum through their potentiation of hCG-stimulated progesterone production (Fig. 61).

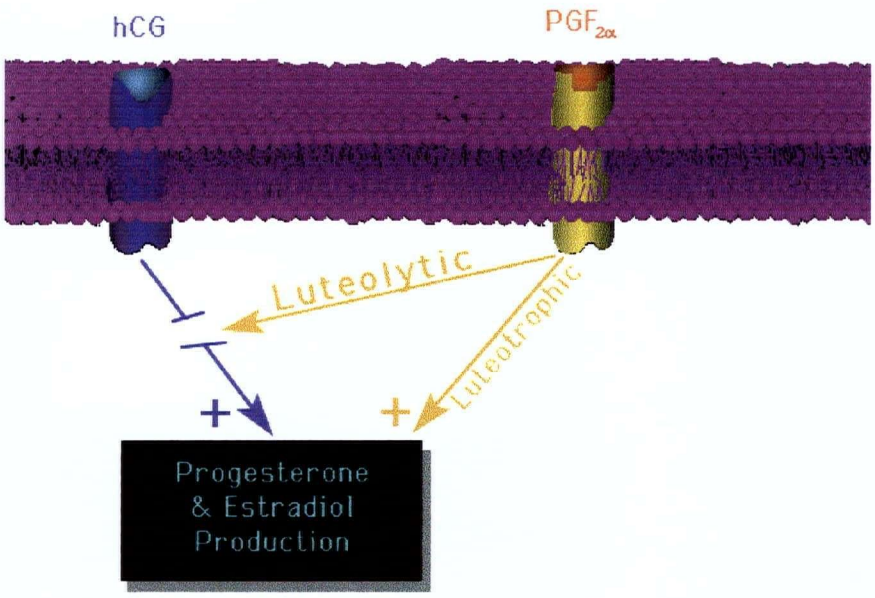


Figure 61. Dual (bimodal) actions of Prostaglandin $F_{2\alpha}$ on progesterone and estradiol production, in human luteal cells.

B. Interaction of $PGF_{2\alpha}$ with GnRH

Progesterone Response

This study examined the interactions of GnRH and $PGF_{2\alpha}$ in the presence and absence of hCG. In general, at the concentrations tested, neither GnRH nor $PGF_{2\alpha}$ altered progesterone or estradiol production in D_1 cultures. However, when GnRH and $PGF_{2\alpha}$ were co-applied, a significant stimulation of progesterone and estradiol production was seen. Furthermore, in D_8 cultures where a weak luteotrophic action was seen with $PGF_{2\alpha}$ treatment, GnRH potentiated $PGF_{2\alpha}$-stimulated progesterone production, but was again ineffective on its own. These results suggest that $PGF_{2\alpha}$ requires GnRH as a permissive factor in order for its luteotrophic action to be present, and that GnRH on its own is not a luteotrophin. The ability of $PGF_{2\alpha}$ to stimulate progesterone production in D_8 cultures suggests that endogenous GnRH may be present. Alternative explanations would be that a second permissive factor exists, or that $PGF_{2\alpha}$ does not require one in later cultures. Interestingly, the magnitude of the response is much greater in D_8 cultures. When viewed in three dimensions, the nature of the interactions between GnRH and $PGF_{2\alpha}$ are intriguing. At optimal $PGF_{2\alpha}$ concentrations, GnRH stimulated progesterone production appears linear and concentration dependent. At optimal GnRH conditions, $PGF_{2\alpha}$-stimulated progesterone production is bell curve-like. One remarkable characteristic of GnRH plus $PGF_{2\alpha}$-mediated progesterone production is the consistency of the response. In this laboratory, the luteotrophic response to $PGF_{2\alpha}$ alone is only present in cells from about 50% of patients, while in these experiments, 100% of patients demonstrated a luteotrophic response with co-application of these two hormones.

In D_1 cultures and at the concentrations tested, GnRH was not luteolytic, although it was in D_8 cultures. Gonadotrophin-releasing hormone potentiated $PGF_{2\alpha}$-mediated luteolysis in D_1 and D_8 pre-cultured human GLCs.

Estradiol Response

Estradiol production was also regulated in a remarkable fashion by the co-application of GnRH and $PGF_{2\alpha}$. In the presence of high concentrations of GnRH, $PGF_{2\alpha}$ linearly and concentration dependently stimulated estradiol production. These effects of GnRH appeared to

be permissive, as $PGF_{2\alpha}$-mediated stimulation of estradiol production was not present in the absence of GnRH. Moreover, the linear stimulation of estradiol production by $PGF_{2\alpha}$ (in the presence of high concentrations of GnRH) appeared to be similar to the response seen in eight-day cultures (in the absence of exogenous GnRH; not shown). Perhaps the levels of endogenous GnRH in the culture system increase with culture time, with the effect of modulating the response over culture-time. Gonadotrophin-releasing hormone alone, significantly stimulated estradiol production in a bell curve-like manner. However, in the presence of high concentrations of $PGF_{2\alpha}$, GnRH-mediated stimulation of estradiol production shifted from being bell curve-like to linear. The effects of $PGF_{2\alpha}$ on the response to GnRH were modulatory rather than permissive.

Implications

The striking difference in the progesterone and estradiol responses to $PGF_{2\alpha}$ and/or GnRH, may play an important role in the regulation of the luteal phase or even the menstrual cycle. If one superimposes the three dimensional plots of these two hormones, one can see that as the concentrations of $PGF_{2\alpha}$ and/or GnRH change, so does the ratio of progesterone to estradiol production. For example, at high concentrations of GnRH and middle concentrations of $PGF_{2\alpha}$ estradiol production is low relative to progesterone production, which is at its highest. However, at high concentrations of GnRH and $PGF_{2\alpha}$ estradiol production is at its highest, while progesterone production is at its lowest level. Thus, due to the co-operative nature of $PGF_{2\alpha}$ and GnRH, subtle changes in the concentrations of these hormoens can have profound effects on the ratio of progesterone to estradiol production. With this in mind, it is interesting to note that under conditions conducive to a luteotrophic response (ie. high progesterone production; $PGF_{2\alpha}$ 10^{-9} and GnRH 10^{-5} M) estradiol production is relatively low, while under conditions optimal for luteolysis (ie. low progesterone production; PGF 10^{-6} and GnRH 10^{-5} M) estradiol production is high.

The present results further support the hypothesis that $PGF_{2\alpha}$ and GnRH have very similar and/or complementary roles in the ovary. Teleologically speaking, the advantages of a dual hormone system over a single hormone system seem obvious. Should one system be deficient or fail, the second system would provide a backup. Conversely, when both systems are

working amplification and fine tuning of the signal are improved. Interestingly, a positive feedback loop may exist in this system, as GnRH has been shown to stimulate arachidonic acid and PGE_2 from the luteal cell, and PGF production in the placenta [Kawai and Clark, 1985 and 1986; Hillensjo et al., 1982; Siler et al., 1986]. It is unknown if $PGF_{2\alpha}$ stimulates GnRH production in these cells. The bell curve-like response to $PGF_{2\alpha}$ could act as a fine tuning or switching mechanism, allowing a luteotrophic response to turn into a luteolytic one should $PGF_{2\alpha}$ levels increase much beyond nM concentrations. This bimodal response to prostaglandins is not unique to the corpus luteum [Cohen and Rimon, 1992; Sano and Shichi, 1993; Puschel et al., 1993; Hargrove et al., 1975]. For example, in the rat and rabbit testicular tissues, bimodal responses to PGE_2 and PGE_1 have been reported, respectively [Hillensjo et al., 1982; Sano and Shichi, 1993]. Rat epididymal adipocytes displayed a PGE_2-mediated inhibition and stimulation of cAMP at concentrations of 10 mM and >10 mM, respectively. In the rabbit, testicular contractions were stimulated and inhibited by PGE_1, at concentrations of 1–10 nM and 100 nM, respectively. Non-reproductive tissues also have been shown to exhibit bimodal responses to prostaglandins [Siler et al., 1986; Cohen and Rimon, 1992]. In rat hepatocytes, PGE_2 can act in a glycogenolytic and in a antiglycogenolytic fashion at concentrations of 10 μM and 1 nM, respectively. As mentioned previously, these glycogenolytic and antiglycogenolygic actions are likely mediated through the inositol triphosphate and cAMP pathways, respectively.

Controversy over which effect (luteotrophic or luteolytic) is physiological are bound to arise from these results and those of others. The reported Kd for the $PGF_{2\alpha}$ receptor is in the nano-molar range [Lake et al., 1994], close to the concentrations at which the luteotrophic response is present. It is likely that both responses are physiological and that their temporal relationship to luteolysis and early pregnancy is important. As mentioned above, studies have demonstrated the predominance of the luteotrophic response in the mid-luteal phase [Khan et al., 1989; Richardson and Masson, 1980; Michael and Webley, 1993], corresponding well with a potential role in the promotion of early pregnancy. In support of this notion, there have been reports of enhanced progesterone production in the presence of hCG plus $PGF_{2\alpha}$ compared with hCG alone [Suginami et al., 1976], as well as studies that have demonstrated the abrogation of $PGF_{2\alpha}$-mediated luteolysis when cellular exposure to hCG or prolactin preceeds $PGF_{2\alpha}$ exposure [Harris and Murphy, 1981; Suginami et al., 1976; Michael and Webley, 1991b].

Two models by which GnRH may be permissive or potentiatory with respect to $PGF_{2\alpha}$-mediated steroidogenesis are depicted in Figures 62 and 63. In short, GnRH may provide a missing component in a $PGF_{2\alpha}$-affected signal transduction system, or it may promote the production of *de novo* $PGF_{2\alpha}$. Further treatment of this subject may be found below in the signal transduction section (p. 132).

With enhanced magnitude and consistency of both the luteotrophic and luteolytic actions of $PGF_{2\alpha}$ and GnRH when co-applied, the potential for improving the clinical applications of these two hormones exists. Currently, potent, long-lasting GnRH analogues have been applied to numerous therapeutic applications in the female including: contraception; treatment of central precocious puberty; and sex steroid-dependent benign and malignant diseases of the reproductive organs [Molcho et al., 1984; Bhasin et al., 1984; Nillius, 1985]. One of the most common uses of GnRH analogues is the down-regulation of pituitary function in preparation for super-ovulation as part of an *in vitro* fertilization protocol. Conversely, attempts to inhibit luteal function and induce luteolysis or early abortion with GnRH have not been very successful [Nillius, 1985]. The contraceptive effects of GnRH appear to be safe, reliable and reversible in women [Nillius, 1985].

Experimental Model

When examining the interactions of two or more hormones, the author has found multi-concentration experiments to be much more revealing than single concentration studies. The difficulties with these experimental models lie in visualization of the results. Plotting data in three dimensions with the two interacting agents on the horizonal axes and the response on the vertical axis simplifies interpretation of the results. Further enhancement of visualization of the results with a contour plot of the data was achieved. This design and the improved speed of modern computers in plotting such data has revealed a more complex interaction between these two hormones than would be visible with a standard two dimensional experimental design. Certainly numerous other hormones are interacting in similar or even more complex manners.

Summary

These studies reveal the complex luteolytic and luteotrophic actions of GnRH and $PGF_{2\alpha}$.

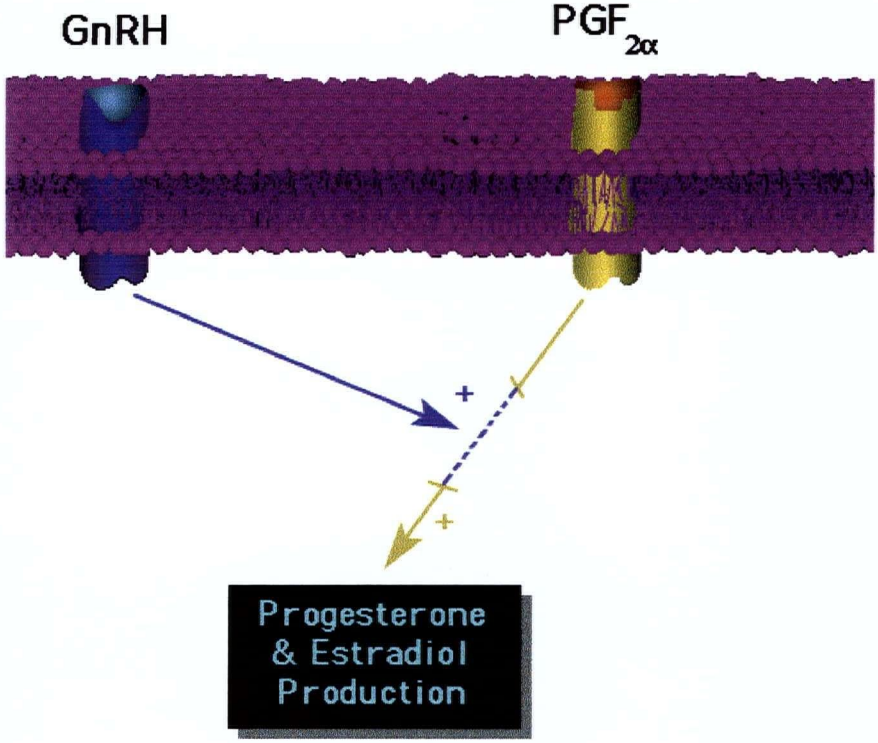


Figure 62. Gonadotrophin-releasing hormone (GnRH) acts as a permissive or potentiatory factor for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-mediated luteotrophic and luteolytic effects, respectively. This model suggests that GnRH may provide a missing signal transduction factor, thus completing or enhancing the signal transduction pathways by which $PGF_{2\alpha}$ functions.

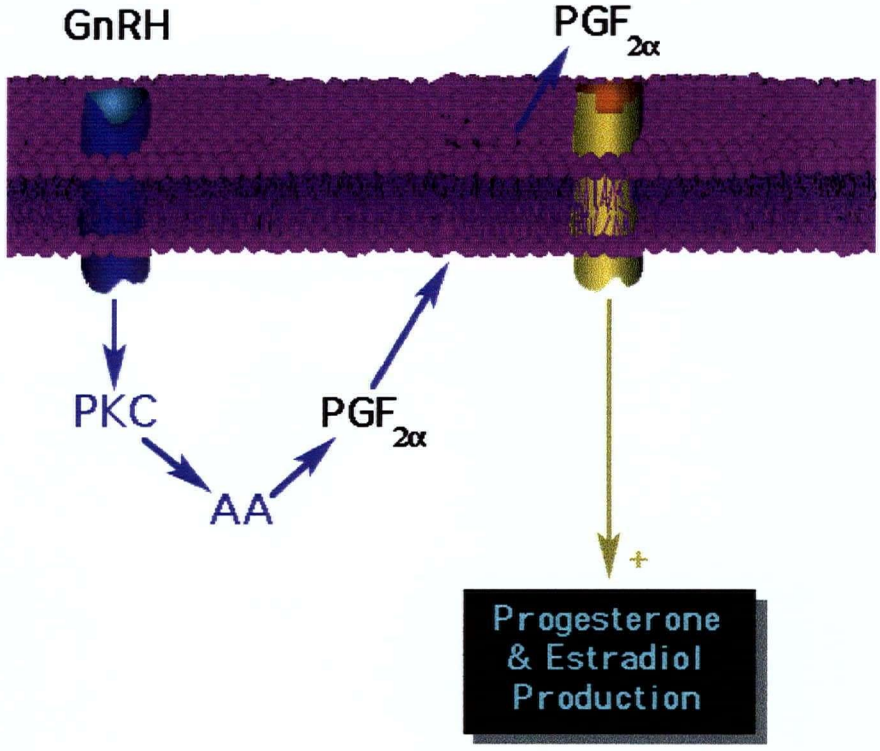


Figure 63. Gonadotrophin-releasing hormone (GnRH) acts as a permissive or potentiatory factor for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-mediated luteotrophic and effects luteolytic, respectively. This model suggests that GnRH may stimulate *de novo* synthesis of $PGF_{2\alpha}$, through a PKC-dependent stimulation of arachidonic acid (AA) production, as is seen in some other systems [Smith and Borgeat 1988].

C. Interaction of $PGF_{2\alpha}$ with PGE_2.

In eight-day precultured GLCs, both $PGF_{2\alpha}$ and PGE_2 stimulated progesterone production in a bell curve-like manner. As with previous experiments $PGF_{2\alpha}$ exerted its maximal luteotrophic effect at a concentration of 1 nM. Interestingly, the bell curve-like stimulation mediated by PGE_2 was shifted to the right (when compared to that of $PGF_{2\alpha}$) such that maximal stimulation of progesterone production was seen at a concentrations of 10 to 100 nM. In both cases, maximal luteotrophic effects were present at or near the Kds of their receptors [Abramovitz et al., 1994; Abramovitz et al., 1994b; Lake et al., 1994].

The combination of these hormones resulted in an elaborate regulation of progesterone production whereby the bell curve was inverted. There are a number of possible explanations for the complexity of this regulation, including: 1) the non-standard saturation kinetics of PLA_2; 2) product inhibition of PGE_2 production by $PGF_{2\alpha}$; 3) receptor cross reactivity; 4) the opposing actions of these hormones on common signal transduction pathways; and 5) the combination $PGF_{2\alpha}$-mediated luteolytic and luteotrophic effects in the presence of PGE_2. It is likely that all of these factors play a role in these findings.

The physiological significance of these findings is not obvious at first glance. However, these results provide a potential mechanism for the changing functional role of the corpus luteum as luteinization progresses. Changing the ratio of $PGF_{2\alpha}$ to PGE_2 can profoundly alter progesterone production, a result seen in other reports which compared luteal cells in early, mid and late luteal stages [Pathwardhan and Lanthier, 1985]. Although complex in nature, these results should not be ignored as the ratio of $PGF_{2\alpha}$ to PGE_2 is known to change *in vitro*. Taken with the confounding effects of $PGF_{2\alpha}$ and GnRH, there exists more than adequate room for explaining the changing responses of granulosa and luteal cells with differentiation. Further, these results suggest that $PGF_{2\alpha}$ interactions with PGE_2 form a sophisticated means of regulating progesterone production in human granulosa-luteal cells.

D. Signal Transduction of $PGF_{2\alpha}$-Mediated Luteolysis

This study has examined the signal transduction pathways utilized in the anti-gonadotrophic (or luteolytic) actions of $PGF_{2\alpha}$ in the human granulosa-luteal cell. Specifically, the ability of $PGF_{2\alpha}$ to inhibit both hCG- and isoproterenol-stimulated progesterone production was examined, as well as hCG-stimulated estradiol production. Furthermore, the signal transduction pathways involved in this effect were examined with pertussis- and cholera-toxin, as well as with forskolin and db-cAMP.

As seen above, the basal responses to $PGF_{2\alpha}$ were quite variable (Fig. 47, 49, and 51-53). This may be due to differences in the endogenous levels of $PGF_{2\alpha}$ within the culture media, differences in cellular differentiation state or differences in endogenous levels of interacting hormone levels. Further studies are underway to determine the exact nature of this phenomenon.

Pertussis Toxin Sensitive G-Protein

It was found that $PGF_{2\alpha}$ is exerting its anti-gonadotrophic (specifically anti-hCG) actions through a pertussis toxin sensitive G-protein. These data were supported by the ability of $PGF_{2\alpha}$ to inhibit CTX-stimulated progesterone and estradiol production, and by the reversal of this inhibition by the addition of PTX. These data also suggest that $PGF_{2\alpha}$ may exert its anti-gonadotrophic actions at an early step in the signal transduction cascade.

Reports in the literature support the potential role of G-proteins in the signal transduction of $PGF_{2\alpha}$. Firstly, human granulosa-luteal cells have been examined immunocytochemically to reveal a number of G-protein alpha subunits, including, $G_{\alpha s}$, $G_{\alpha i3}$, $G_{\alpha i1,2}$ and $G_{\alpha p}$ (namely, $G_{\alpha q}$ and $G_{\alpha 11}$), while G_o was undetectable by three different antibodies [Lopez et al., 1995]. Intracellular cAMP levels in human granulosa cells appear to be regulated by the ratio of $G_{\alpha s}$ and $G_{\alpha i}$-subunits, while $G_{\alpha q,11}$ and $G_{\alpha i}$ levels regulate the accumulation of inositol phosphates [Lopez et al., 1995]. Coupling of one or both of these identified $G_{\alpha i}$-subunits to the $PGF_{2\alpha}$-receptor could explain the PTX sensitivity of the anti-gonadotrophic action of $PGF_{2\alpha}$, as well as the regulation of both cAMP and inositol phosphates by $PGF_{2\alpha}$.

Further supporting a role for G-proteins in the signal transduction of $PGF_{2\alpha}$ are the sequences and predicted structure of the cloned prostanoid receptors. All of these receptors possess the seven-transmembrane domain structure characteristic of G-protein coupled receptors [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993]. Additionally, the human EP_3-family of receptors is capable of inhibiting cAMP production through a pertussis toxin-sensitive G-protein [An et al., 1994]. It is not known if $PGF_{2\alpha}$ is acting through single or multiple G-proteins, as is seen in the actions of gonadotrophin-releasing hormone (GnRH) in the gonadotrope [Hawes et al., 1993].

Thus far, $PGF_{2\alpha}$ has been demonstrated to lower gonadotrophin- and prostaglandin E_2-stimulated rises in cAMP, as well as increase intracellular calcium and inositol phosphates [Davis et al., 1989; Currie et al., 1992; Pepperell et al., 1989; Lahav et al., 1987]. The DP, IP and EP_3-family of receptors are all coupled to cAMP regulation [Adam et al., 1994; Boie et al., 1994 and 1995; An et al., 1994], while the DP, FP, EP_1 and EP_3-family of receptors are coupled to rises in intracellular calcium [Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1995; Funk et al., 1993; An et al., 1994]. It is unknown if the actions of $PGF_{2\alpha}$ are exerted through single or multiple–receptors. With $PGF_{2\alpha}$ and PGE_2 both being present and active in the human granulosa and luteal cells [Grinwich et al., 1976; Richardson and Masson, 1980; Pathwardhan and Lanthier, 1985; Satoh et al., 1981; Watson et al., 1979], it is probable that multiple prostanoid receptors exist in these cells. Furthermore, the currently cloned prostanoid receptors all possess varying degrees of cross–reactivity with PGE_2 and $PGF_{2\alpha}$ [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993; An et al., 1994].

Although the present results indicate that the anti-gonadotrophic effects of $PGF_{2\alpha}$ are due to a pertussis toxin sensitive G-protein, it is unclear if they are mediated through $G_{\alpha i}$, $G_{\alpha p}$ or both. The G-protein alpha-subunit designated $G_{\alpha p}$ is not a single G-protein but is, in fact, a family of G-proteins capable of activating phosphoinositide phosphodiesterase [Cockcroft and Stutchfield, 1988]. More than one G_p pathway often exists within a single cell type, with distinct proteins coupling different receptors to phosphatidyl inositide hydrolysis selectively, thus allowing for regulation of the magnitude of phosphatidyl inositide hydrolysis [Ashkenazi et al., 1989]. Within the Gp family of G-proteins there exists two sub-families, a pertussis toxin-sensitive and a pertussis toxin-insensitive G_p, both of which are involved in PLC regulation [Martin et al., 1991]. For example, bovine adrenal fasiculata cells possess angiotensin-II

receptors which are coupled to the phosphoinositide pathway through pertussis toxin-sensitive and insensitive G_p proteins [Langois et al., 1990]. This example demonstrates the ability of a single receptor to be coupled to multiple-forms of G_p, providing for a complex regulation of the phosphoinositide pathway.

If the anti-gonadotrophic effects of $PGF_{2\alpha}$ are mediated by $G_{\alpha i}$, this would probably be through a direct effect of $G_{\alpha i}$ on adenylate cyclase and/or through the 'mopping-up' of $G_{\alpha s}$-subunits by free beta/gamma-subunits freed when $G_{\alpha i}$ was released. Alternatively, if the anti-gonadotrophic effects of $PGF_{2\alpha}$ are mediated through a pertussis toxin sensitive $G_{\alpha p}$, it is likely that inhibition of the cAMP pathway would be through elevated levels of inositol phosphates, calcium, diacylglyceride and PKC activity and through the actions of these messengers on the cAMP pathway. In the sheep, it has been demonstrated that elevated levels of phospholipase-C activity involves a pertussis toxin-sensitive protein [McCann and Flint, 1993]. It is also possible that the $PGF_{2\alpha}$-receptor is coupled to more than one G-protein,. Finally it has been suggested that a single $G_{\alpha i}$-like $G_{\alpha p}$ protein may be capable of multiple actions [Magnaldo et al., 1988]. In hamster fibroblasts, thrombin is capable of inhibiting adenylate cyclase via a G-protein, while the G-protein mediated-activation of PKC causes a stimulatory effect on adenylate cyclase. This indirect stimulatory effect is exerted by PKC action directly on an element of the adenylate cyclase-$G_{\alpha s}$ complex [Magnaldo et al., 1988]. If a similar mechanism existed in the human granulosa-luteal cell, this might help to explain the transient stimulatory effects of $PGF_{2\alpha}$ on basal progesterone production.

In the gonadotrope, it has been demonstrated that GnRH may exert its actions through as many as three G-proteins [Hawes et al., 1993]. Briefly, GnRH has been demonstrated to stimulate IP production through a PTX-sensitive G-protein (Gp), while a distinct CTX-sensitive G protein can sensitize the gonadotrope to luteinizing hormone (LH) release through cAMP. Finally, a third CTX/PTX-insensitive G-protein can mediate LH release. Furthermore, there has been the suggestion of cross-talk between the CTX-sensitive G-protein and the PKC pathway [Barnes and Conn, 1993]. In view of the remarkable similarities (signal transduction and steroidogenic effects) between GnRH and $PGF_{2\alpha}$ [Quirk et al., 1995; Leung and Steele, 1992; Stoljelkovics et al., 1994] and their receptors [Lake et al., 1994; Abramovitz et al., 1994; Stoljelkovics et al., 1994] in the ovary, similar complexities may play a role in the signal transduction of the actions of $PGF_{2\alpha}$ in the human granulosa-luteal cells.

Adenylate Cyclase and cAMP

In these studies, $PGF_{2\alpha}$ inhibited forskolin- but not db-cAMP-stimulated progesterone production from human granulosa-luteal cells, suggesting that it is exerting its actions at or above the level of gonadotrophin-dependent adenylate cyclase. In previous studies, forskolin-stimulated progesterone production from large luteal cells (bovine CL) was inhibited by $PGF_{2\alpha}$ only in the presence of endothelial cells [Girsh et al., 1995]. This action was attributed to the secretion of PGI_2 by endothelial cells. In contrast to the present results, several other studies in rat and bovine luteal cells, have demonstrated the inhibition of progesterone production by $PGF_{2\alpha}$ at sites distal to adenylate cyclase [Rajkumar et al., 1988; Benhaim et al., 1987; Dorflinger et al., 1983]. This inhibition may be mediated by a reduction in the sensitivity of the cells to cAMP.

In light of the irreversibility of forskolin-activation of adenylate cyclase it is unlikely that $PGF_{2\alpha}$ is exerting it's inhibitory actions solely on adenylate cyclase. These actions may be exerted in part through a phosphodiesterase family member (Michael and Webley 1991a).

The ß-adrenergic receptor, one of the most studied and understood of receptors, has been well established as a seven transmembrane, G-protein coupled receptor which activates the production of cAMP from adenylate cyclase [Briggs 1982; Barak et al., 1995; O'Dowd et al., 1988]. Several studies have demonstrated that isoproterenol (a ß-adrenergic agonist) is capable of stimulating cAMP and progesterone production from granulosa and luteal cells in non-primate species [Leung, 1985]. However, thus far there have been conflicting reports regarding the effects of isoproterenol on progesterone production and adenylate cyclase activity in the granulosa-luteal cells of the human [Fohr et al., 1993; Casper and Cotterell, 1984; Hellensjo et al., 1985]. This study demonstrates that isoproterenol is capable of stimulating progesterone production in the human granulosa-luteal cell, and that this stimulation may be inhibited by $PGF_{2\alpha}$. The ability of $PGF_{2\alpha}$ to inhibit isoproterenol-stimulated estradiol production was not examined in these studies, although isoproterenol was found to stimulate estradiol production (not shown).

Protein Kinase C

As mentioned above, previous studies have demonstrated the ability of PKC inhibitors to partially inhibit $PGF_{2\alpha}$-mediated luteolysis. These studies have confirmed this result. The

inhibitory actions of PKC inhibitors are not as complete as those of PTX, suggesting that PKC inhibitors are blocking the effects of $PGF_{2\alpha}$ through an indirect means. Based on the known pathways by which prostaglandin production is stimulated in other models [Smith and Borgeat 1988], the author would like to suggest that endogenous $PGF_{2\alpha}$ production may be stimulated by the calcium-diglyceride-PKC pathway in these cells. This would help to explain the partiality of the PKC inhibitory effect on $PGF_{2\alpha}$-mediated luteolysis, as *de novo* prostaglandin synthesis would be blocked, although existing $PGF_{2\alpha}$ could still be effective until degraded. Moreover, it would provide a mechanistic explanation for the ability of GnRH to potentiate or permit the effects of $PGF_{2\alpha}$, as GnRH is known to stimulate the calcium-diglyceride-PKC pathway as well (see Fig. 64). Additionally, the known stimulation of this signal transduction pathway by $PGF_{2\alpha}$ could provide a positive feedback mechanism. In support of this idea is the ability of indomethacin to block the luteotrophic effects of $PGF_{2\alpha}$. As indomethacin is a blocker of *de novo* prostaglandin production, this effect suggests that in order for $PGF_{2\alpha}$ to exert its actions in these cells a feedback loop is necessary. Given the short half-life of $PGF_{2\alpha}$, this would make sense.

De Novo Protein Synthesis

Interestingly, the effects of $PGF_{2\alpha}$ are reported to be dependent on *de novo* protein synthesis, as actinomycin-D blocks them [Fitz et al., 1993]. It would not be surprising if this *de novo* protein is either an eicosogenic enzyme or GnRH.

Summary

The anti-gonadotrophic actions of $PGF_{2\alpha}$ are mediated through a pertussis-toxin sensitive G-protein (possibly G_i, G_p or both; Fig. 65). Prostaglandin $F_{2\alpha}$ is capable of inhibiting progesterone production in response to hCG (Fig. 66), isoproternol (Fig. 66), CTX (Fig. 67A) and forskolin (Fig. 67B), but not db-cAMP (Fig. 67), strongly suggesting that $PGF_{2\alpha}$ is exerting its anti-gonadotrophic actions at or above the level of adenylate-cyclase. Further supporting this conclusion is the ability of PTX to reinstate the stimulatory actions of CTX following $PGF_{2\alpha}$ administration. There exists the potential that these actions are exerted through a member of the phosphodiesterase family of enzymes.

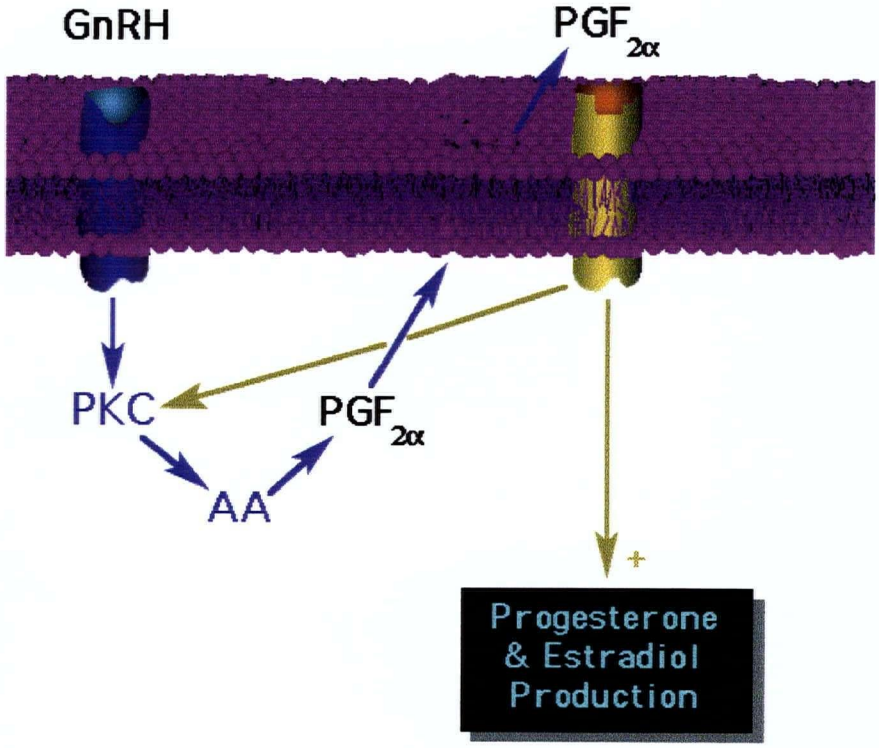


Figure 64. Proposed positive feedback loop for prostaglandin $F_{2\alpha}$ synthesis ($PGF_{2\alpha}$). Gonadotrophin-releasing hormone (GnRH) and $PGF_{2\alpha}$ are both known to stimulate the calcium-diglyceride-protein kinase C (PKC) pathway. Moreover, PKC pathway is reported to stimulate *de novo* prostaglandin synthesis in some systems. Thus, there exists the possiblity that $PGF_{2\alpha}$ and GnRH may provide postive feedback on *de novo* $PGF_{2\alpha}$ synthesis in the human granulosa-luteal cell.

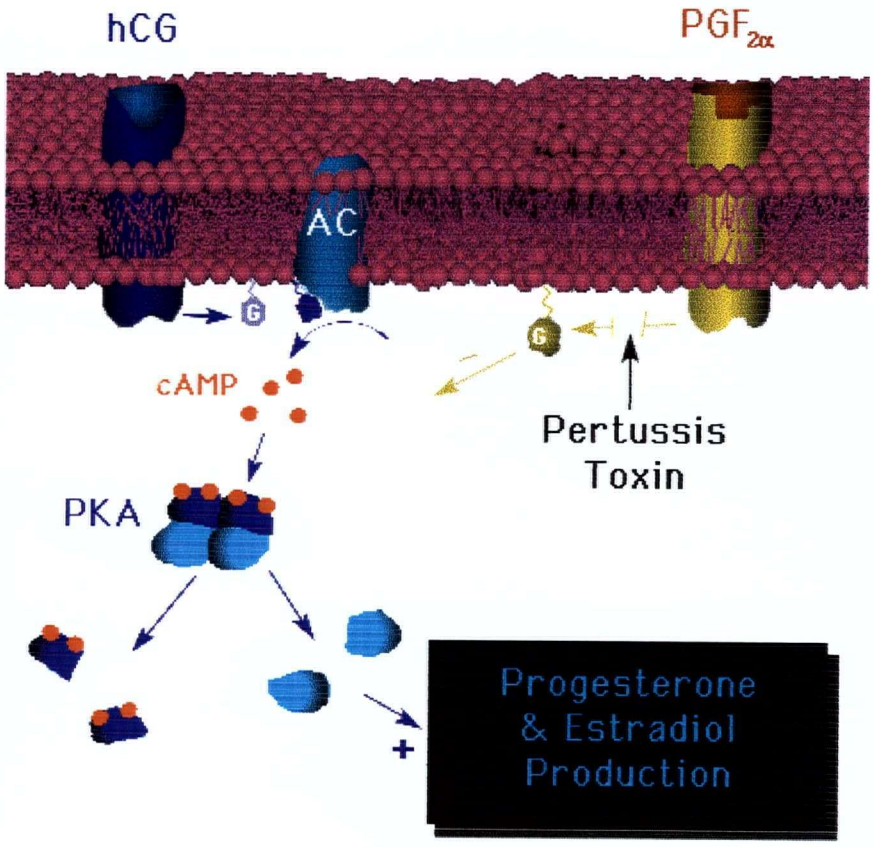


Figure 65. Pertussis toxin blocks prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-mediated inhibition of human chorionic gonadotrophin (hCG)-stimulated steroidogenesis in the human luteal cell. G - stimulatory G-protein; G - pertussis toxin sensitive G-protein; AC - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A

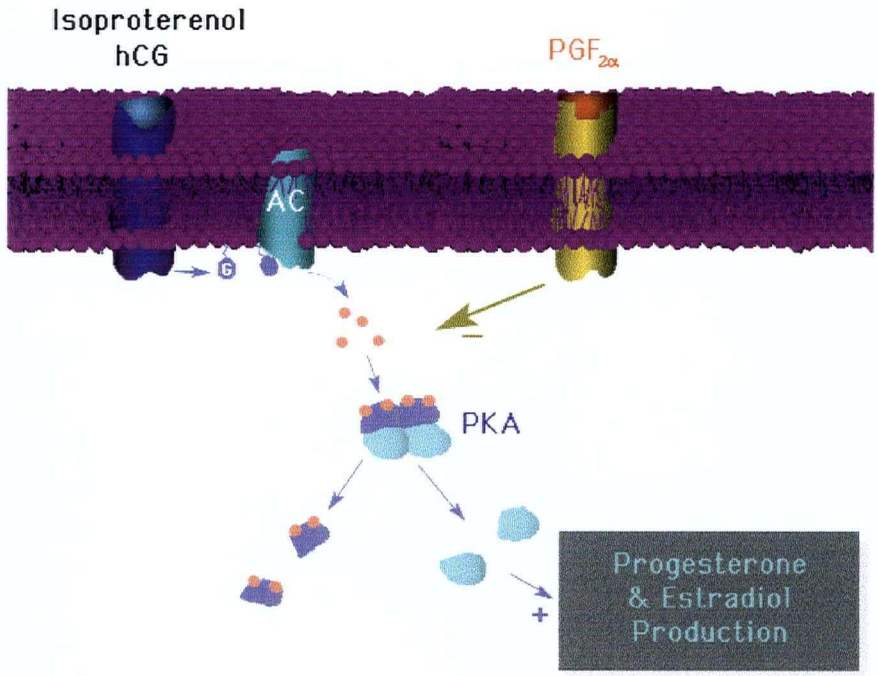


Figure 66. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-mediated inhibition of human chorionic gonadotrophin (hCG)- and isoproterenol-stimulated steroidogenesis, in the human granulosa-luteal cell. G - stimulatory G-protein; AC - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A

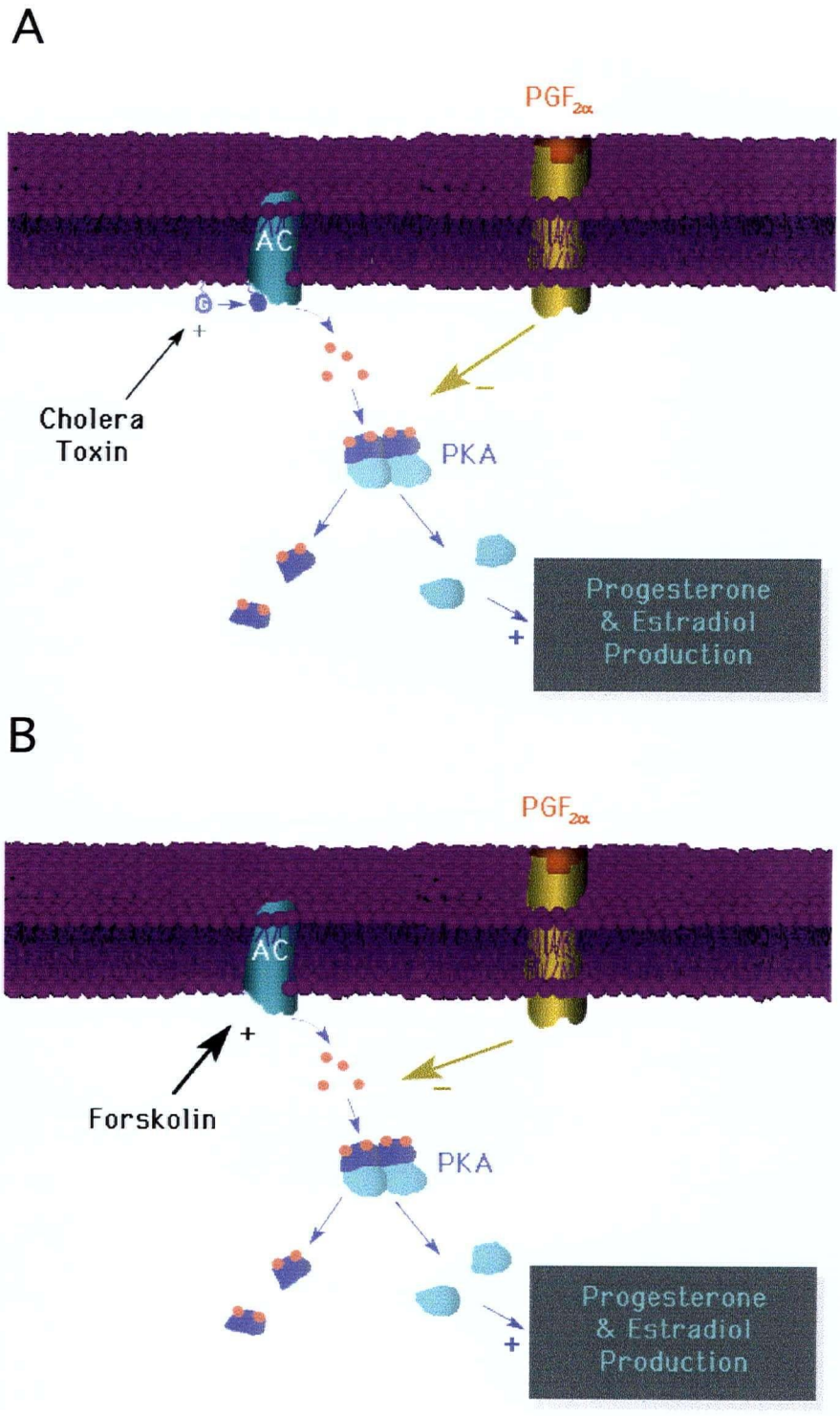


Figure 67. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-mediated inhibition of cholera toxin (**A**) and forskolin (**B**) stimulated progesterone and estradiol production in human granulosa-luteal cells. G - stimulatory G-protein; AC - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A

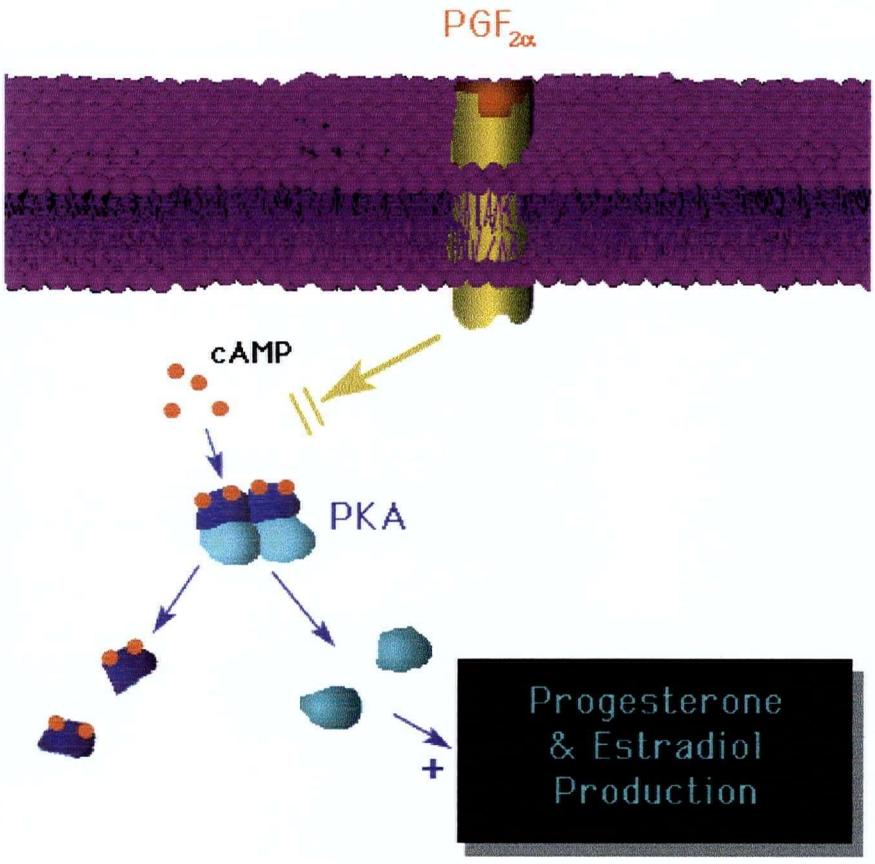


Figure 68. The inability of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to inhibit dibutryl cyclic adenosine monophosphate (cAMP) stimulated progesterone and estradiol production in human granulosa-luteal cells. PKA - protein kinase A

E. Regulation of $PGF_{2\alpha}$-R mRNA

An inverse relationship between progesterone production and $PGF_{2\alpha}$-R mRNA levels was revealed in the present studies. Human chorionic gonadotrophin and $PGF_{2\alpha}$ both inhibited $PGF_{2\alpha}$-R mRNA levels. Maximal inhibition of $PGF_{2\alpha}$-R mRNA levels was seen at 1 nM $PGF_{2\alpha}$ in the presence of hCG. As this receptor is only recently cloned in the human and rat [Abramovitz et al., 1994; Lake et al., 1994], there exists only one other report of $PGF_{2\alpha}$-R mRNA regulation in the literature. Moreover, the effects of $PGF_{2\alpha}$ on $PGF_{2\alpha}$-R mRNA levels have not been examined. However, the effects of hCG have been examined [Ristimaki et al., 1997]. This report demonstrated an hCG-mediated upregulation of $PGF_{2\alpha}$-R mRNA levels– a result differing from the present results. The difference between these two reports may be explained by the fact that these experiments were performed on cells of different culture periods.

Inhibition of $PGF_{2\alpha}$-R mRNA and presumably $PGF_{2\alpha}$-R would reduce the effectiveness of $PGF_{2\alpha}$-mediated luteolytic effects. Thus, the inverse bell curve-like autoregulation of $PGF_{2\alpha}$-R mRNA by $PGF_{2\alpha}$ may explain its bell curve-like effects on progesterone production. Notably, maximal stimulation of progesterone production in the presence of hCG and $PGF_{2\alpha}$ (1 nM) occurred when $PGF_{2\alpha}$-R mRNA levels were at their lowest. Thus, rather than potentiating hCG in a true sense, $PGF_{2\alpha}$ is inhibiting its own luteolytic effects allowing more effective stimulation by gonadotrophins. The mechanism by which $PGF_{2\alpha}$ autoregulates its receptor mRNA needs to be studied further.

In summary, $PGF_{2\alpha}$ negatively autoregulates its receptor mRNA. Moreover, $PGF_{2\alpha}$ feeds back on its steroidogenic effects through this autoregulation.

VIII – SYNOPSIS

The aforementioned studies examined the effects of prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) on progesterone and estradiol production, as well as DNA and $PGF_{2\alpha}$-R mRNA levels in the human granulosa-luteal cell (GLC). Additionally, the interactions of $PGF_{2\alpha}$ with human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone (GnRH) and prostaglandin E_2 (PGE_2) were examined with respect to progesterone and estradiol production. In one study, cells were collected from small (<12 mm) and large (>12 mm) follicles separately, permitting the examination of follicle size-dependent alterations in steroidogenisis. Pharmacological techniques were utilized to elucidate the signal transduction pathways involved in the anti-gonadotrophic effects of $PGF_{2\alpha}$. Moreover, these experiments were performed on one-(D_1), eight-(D_8) and/or twelve- to fourteen-day ($D_{12\text{-}14}$) cultured GLCs in order to reveal culture time-dependent alterations in cellular response.

Briefly, GLCs collected from patients undergoing *in vitro* fertilization (IVF) were cultured for the time periods described above, followed by a 24 h treatment period, after which media were collected and assayed for progesterone and estradiol while cells were extracted for DNA or total RNA.

A. Basic Physiological Responses to $PGF_{2\alpha}$

Human GLCs undergo morphological luteinization and then luteolysis with increasing time in culture. Culture-time, concentration and/or follicle-size dependent alterations in $PGF_{2\alpha}$- and GnRH-mediated regulation of human GLC steroidogenesis in the presence and/or absence of human chorionic gonadotrophin (hCG) were investigated. This study clearly demonstrated functional differentiation of human granulosa-luteal cells in culture.

Progesterone production in response to $PGF_{2\alpha}$ changed with culture-time from inhibition to stimulation in D_1 and $D_{12\text{-}14}$ cultured GLCs, respectively. Cells at D_8 of culture were in a state of transition, with inhibition, stimulation or intermediate responses being possible. Similarly, estradiol responses changed from no response to a stimulatory response in D_1 and D_8 cultured GLCs, respectively. DNA levels were unaltered by $PGF_{2\alpha}$ treatment.

In the presence of hCG similar culture-time dependent changes were observed. $PGF_{2\alpha}$ (10^{-6} M) inhibited hCG-stimulated progesterone production in D_1 and D_8, but not in $D_{12\text{-}14}$ cultured GLCs. In contrast, $PGF_{2\alpha}$ (10^{-9} M) potentiated hCG-stimulated progesterone production in D_8 and $D_{12\text{-}14}$, but not in D_1 cultured GLCs. A similar trend was seen with estradiol production. Human CG significantly stimulated progesterone and estradiol production from D_1 cultured GLCs collected from large follicles (> 12 mm), while it did not in cells collected from small follicles (< 12 mm). Consequently, $PGF_{2\alpha}$ significantly inhibited hCG-stimulated progesterone and estradiol production in GLCs collected from large, but not small follicles. Human CG stimulated progesterone production was inhibited by high concentrations of GnRH (10^{-6} M) in D_1 and D_8 cultured GLCs. Human CG-stimulated progesterone production was inhibited by high concentrations of GnRH (10^{-6} M) in D_1 and D_8 cultured GLCs.

In a fashion similar to $PGF_{2\alpha}$, GnRH (10^{-9}) was capable of potentiating hCG-stimulated stimulated progesterone production in D_8 human GLCs. Similar results were seen for estradiol production in D_8 GLCs. DNA levels were unaltered by these treatments.

B. Confounding Interactions of $PGF_{2\alpha}$ with GnRH

A second study examined the effects of $PGF_{2\alpha}$ and GnRH and their interactions on progesterone- and estradiol-production from D_1 and D_8 cultured human GLCs. In a preliminary experiment, GLCs were treated with vehicle, $PGF_{2\alpha}$ (10^{-9} M), GnRH (10^{-6} M) or $PGF_{2\alpha}$ plus GnRH in the absence or presence of hCG. It was demonstrated that $PGF_{2\alpha}$ and GnRH alone had no significant effect on progesterone or estradiol production in D_1 GLCs. However, the combination of $PGF_{2\alpha}$ plus GnRH caused a significant stimulation of progesterone and estradiol production. $PGF_{2\alpha}$ partially inhibited hCG-stimulated progesterone- and estradiol-production. Conversely, GnRH did not inhibit hCG-stimulated progesterone- or estradiol-production, although it did potentiate $PGF_{2\alpha}$-mediated inhibition of hCG-stimulated steroidogenesis.

In a second experiment (n=7 patients), vehicle, $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M) and GnRH (10^{-10} to 10^{-5} M) concentration-response curves were crossed into a matrix of 49 separate treatments. Steroidogenic responses were plotted in three dimensions and as a contour map. Moreover, 'slices' of the three dimensional matrix were plotted in two dimenstions and analyzed statistically. Maximal stimulation of progesterone-production (2-3-fold) was seen when medium concentrations of $PGF_{2\alpha}$ interacted with high concentrations of GnRH (10^{-6} to 10^{-5} M). In the

presence of high concentrations of GnRH (10^{-6} M), $PGF_{2\alpha}$ stimulated progesterone production in a bell curve-like fashion, as middle concentrations significantly stimulated while low and high concentrations did not. In the presence of middle concentrations of $PGF_{2\alpha}$ (10^{-9} M), GnRH significantly stimulated progesterone production in a linear concentration-dependent manner. Prostaglandin $F_{2\alpha}$ alone elicited no estradiol response. However in the presence of high concentrations of GnRH (10^{-5} M), a significant concentration-dependent stimulation was seen. Maximal stimulation of estradiol production was seen when high concentrations of $PGF_{2\alpha}$ (10^{-6} M) and GnRH (10^{-5} M) were co-applied. Gonadotrophin-releasing hormone alone stimulated estradiol production in a bell curve-like manner, although in the presence of high concentrations of $PGF_{2\alpha}$ (10^{-6} M), estradiol was stimulated in a linear concentration-dependent manner. Inhibition of cyclooxygenase-I (by indomethacin) prevented the luteotrophic effects of $PGF_{2\alpha}$ in the presence and absence of GnRH in D_1 and D_8 cultured human GLCs.

C. Confounding Interactions of PGF $_{2\alpha}$ with PGE$_2$

In D_8 cultured GLCs, $PGF_{2\alpha}$ and PGE_2 concentration response curves were crossed and treated as with GnRH. Briefly, progesterone was significantly stimulated in a bell curve-like manner by $PGF_{2\alpha}$ with maximal stimulation at 1 nM. A similar response to PGE_2 was seen, although the bell curve was shifted right. Maximal PGE_2-mediated stimulation of progesterone production was seen at 10 to 100 nM. However, in the presence of PGE_2 (10^{-7} M), $PGF_{2\alpha}$ significantly inhibited progesterone production in an inverse bell curve-like manner with maximal inhibition at (10^{-10} to 10^{-8} M, $PGF_{2\alpha}$).

D. Signal Transduction of the Luteolytic Effects of PGF $_{2\alpha}$

The third study utilized the G-protein effectors pertussis toxin (PTX) and cholera toxin (CTX), the ß-adrenergic agonist (and known activator of the cAMP pathway) isoproterenol, forskolin, and the cAMP analogue dibutryl-cAMP (Db-cAMP) to examine the signal transduction pathways involved in the anti-gonadotrophic actions of $PGF_{2\alpha}$ in D_8 GLCs. During the final 18 h of the pre-culture period, the cells were cultured in media or media containing PTX (50 ng/ml) and/or CTX (1 μg/ml). The cells were then treated with vehicle, $PGF_{2\alpha}$ (10^{-6} M), hCG (1 IU/ml) or $PGF_{2\alpha}$ plus hCG in the presence of vehicle, PTX, CTX or PTX plus CTX. In another

experiment, cells were treated with vehicle, $PGF_{2\alpha}$ (10^{-6} M), IsoP (10^{-5} M), or $PGF_{2\alpha}$ plus IsoP. It was demonstrated that $PGF_{2\alpha}$ caused a significant inhibition of hCG stimulated progesterone and estradiol production, and that this inhibition was abolished by PTX. Similarly, cholera toxin-stimulated progesterone and estradiol production was blocked by $PGF_{2\alpha}$, with PTX reversing this effect. Finally, $PGF_{2\alpha}$ also inhibited isoproterenol- and forskolin-stimulated, but not Db-cAMP stimulated progesterone production from eight day cultured human granulosa-luteal cells.

E. Regulation of PGF 2α-R mRNA

Human GLCs (D_1) were exposed to culture media containing either vehicle, hCG (1 IU/ml) or hCG plus $PGF_{2\alpha}$ (10^{-11} to 10^{-6} M), for 24 h. Following the treatment period, cells were extracted for total RNA, which was confirmed to be intact by the presence of 18 and 28S bands revealed by RNA gel electrophoresis. A fixed quantity of mRNA (between 0.5 and 2 μg depending on yield) was reverse transcribed to cDNA and frozen (at -20 C) until used in semi-quantitative PCR. Transcripts for $PGF_{2\alpha}$-R were detected by PCR with two different sets of oligonucleotide primers based on the published human $PGF_{2\alpha}$-R sequence. PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and/or autoradiographed when [^{32}P]dCTP was incorporated. PCR products were confirmed to be those of $PGF_{2\alpha}$-R by size and by Southern blot hybridization with an internal oligonucleotide probe. Photographs and/or autoradiograms of the gels or Southern blots were quantified by densitometry. These experiments were performed a minimum of three times on cells from a minimum of three separate patients. Similar results were seen in all experiments performed.

Prostaglandin $F_{2\alpha}$-R mRNA was significantly down-regulated by hCG when compared to the control. In contrast, progesterone and estradiol production were significantly stimulated by hCG. However, as described above, the addition of both low (10^{-11} M) and high concentrations (10^{-6} M) of $PGF_{2\alpha}$ restored $PGF_{2\alpha}$-R mRNA levels to those of the controls. A corresponding change in progesterone and estradiol levels was seen, such that hCG-stimulated steroidogenesis was significantly inhibited by these concentrations of $PGF_{2\alpha}$. Finally, the strongest effect of $PGF_{2\alpha}$ was seen at a concentration of 10^{-9} M where $PGF_{2\alpha}$-R mRNA was barely detectable. As before, progesterone and estradiol production were inversely related to $PGF_{2\alpha}$-R levels, as hCG-stimulated progesterone and estradiol production were completely restored in the presence of 1 nM $PGF_{2\alpha}$. Messenger RNA levels for the ß-actin gene were unaltered by these treatments.

IX – CONCLUSIONS

Human GLCs undergo morphological luteinization and luteolysis with culture-time. Steroidogenic responses to $PGF_{2\alpha}$ were culture-time and concentration dependent, with $PGF_{2\alpha}$ being either luteolytic or luteotrophic depending on these conditions.

Cyclooxygenase-I inhibition prevented the luteotrophic effects of $PGF_{2\alpha}$, suggesting that *de novo* prostaglandin synthesis is required for this effect. Furthermore, the luteotrophic effects of $PGF_{2\alpha}$ required GnRH as a permissive factor. Additionally, the luteotrophic effects of $PGF_{2\alpha}$ could also be regulated in a complex manner by PGE_2.

The luteolytic effects of $PGF_{2\alpha}$ were mediated through a pertussis-toxin sensitive G-protein (possibly G_i, G_p or both). Prostaglandin $F_{2\alpha}$ inhibited cholera-toxin, isoproterenol and forskolin (but not db-cAMP) stimulated progesterone production, suggesting that this G-protein exerts its actions proximal to PKA (possibly at the level of adenylate cyclase or phosphodiesterase). The luteolytic actions of $PGF_{2\alpha}$ were potentiated by GnRH.

Finally, $PGF_{2\alpha}$ was capable of autoregulating its receptor mRNA levels and, thus, its ability to regulate steroidogenesis in the human GLC. An inverse relationship between $PGF_{2\alpha}$-R mRNA levels and steroidogenesis exists.

REFERENCES

Abayasekara DR, Michael AE, Webley GE, Flint AP. Mode of action of prostaglandin $F_{2\alpha}$ in human luteinized granulosa cells: role of protein kinase C. Mol Cell Endocrinol 1993; 97:81-91.

Abayasekara DR, Jones PM, Persaud SJ, Michael AE and Flint AP, Prostaglandin $F_{2\alpha}$ activates protein kinase C in human ovarian cells, Mol Cell Endocrinol 1993; Feb; 91(1-2):51-7.

Abramovitz M, Boie Y, Nguyen T, Rushmore TH, Bayne MA, Metters KM, Slipetz DM, Grygorczyk R. Cloning and expression of a cDNA for the human prostanoid FP receptor. J Biol Chem 1994; 269:2632-6.

Abramovitz M. Cloning and expression of three isoforms of the human EP_3 prostanoid receptor. Febs Lett 1994b; 338:170-4.

Adam M, Boie Y, Rushmore TH, Muller G, Bastien L, McKee KT, Metters KM, Barak LS, Menard L, Ferguson SS, Colapietro AM, Caron MG. The conserved seven-transmembrane sequence NP(X)2,3Y of the G-protein-coupled receptor superfamily regulates multiple properties of the beta 2-adrenergic receptor, Biochemistry 1995; 34:15407-14.

Advis JP, Simpkins JW, Chen HT, Meites J. Relation of biogenic amines to onset of puberty in the female rat. Endocrinology 1978; 103(1):11-6.

Ackland JF, Schwartz NB, Mayo KE, Dodson RE. Nonsteroidal signals originating in the gonads. Physiological Reviews 1992; 72(3):731-87.

Aksel S, Schomberg DW, Hammond CB. Prostaglandin $F_{2\alpha}$ production by the human ovary. Obstet Gynecol 1977; 50:347-50.

Alecozay AA, Harper MJ, Schenken RS, Hanahan DJ. Paracrine interactions between platelet-activating factor and prostaglandins in hormonally-treated human luteal phase endometrium in vitro. J Reprod Fertil 1991; 91:301-12.

An S, Yang J, So SW, Zeng L, Goetzl EJ. Isoforms of the EP_3 subtype of human prostaglandin E_2 receptor transduce both intracellular calcium and cAMP signals. Biochemistry 1994; 33:14496-502.

Ashkenazi A, Peralta EG, Winslow JW, Ramachandran J, Capon DG. Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. Cell 1989; 56(3):487-93.

Aten RF, Ireland JJ, Weems CW, Behrman HR. Presence of gonadotrophin-releasing hormone-like proteins in bovine and ovine ovaries. Endocrinology 1987; 120(5):1727-33.

Baird DT, Rodger M, Cameron IT, Roberts I. Prostaglandins and antigestagens for the interruption of early pregnancy. J Reprod Fertil Suppl 1988; 36:173-9.

Baldwin DM, Bourne GA, Marshall JC. Pituitary LH responsiveness to GnRH in vitro as related to GnRH receptor number. American Journal of Physiology 1984; 247:E651-6.

Balthazart J, Balthazart RC, Cheng M F. Hormonal control of the gonadal regression and recovery observed in short days in male and female doves. Journal of Endocrinology 1981; 89(1):79-89.

Barak LS, Menard L, Ferguson SS, Colapietro AM, Caron MG. The conserved seven-transmembrane sequence NP(X)2,3Y of the G-protein-coupled receptor superfamily regulates multiple properties of the beta 2-adrenergic receptor. Biochemistry 1995; 34:15407-14.

Bar-Ami S, Gitay-Goren H. Altered steroidogenic activity of human granulosa-lutein cells at different cell densities in culture. Mol Cell Endocrinol 1993; 90(2):157-64.

Barfield RJ, Glaser JH, Rubin BS,Etgen AM. Behavioral effects of progestin in the brain. Psychoneuroendocrinology 1984; 9:217-31[Review].

Barnes SJ, Conn PM. Cholera toxin and dibutyryl cyclic adenosine 3',5'-monophosphate sensitize gonadotrophin-releasing hormone-stimulated inositol phosphate production to inhibition in protein kinase-C (PKC)-depleted cells: evidence for cross-talk between a cholera toxin-sensitive G-protein and PKC. Endocrinology 1993; 133:2756-60.

Barnett JV, Shamah SM, Lassegue B, Griendling KK, Galper JB. Development of muscarinic-cholinergic stimulation of inositol phosphate production in cultured embryonic chick atrial cells. Evidence for a switch in guanine-nucleotide-binding protein coupling. Biochem J 1990; 271(2):443-8.

Bauer DA, Jameson JL. Gonadotrophin-releasing hormone receptor messenger ribonucleic acid expression in the ovary during the rat estrous cycle. Endocrinology 1995; 136(10):4432-8.

Bazer FW, Roberts RM, Thatcher WW. Actions of hormones on the uterus and effect on conceptus development. J Anim Sci 1979; 2:35-45 [Review].

Behrman HR, Luborsky JL, Aten RF, Polan ML, Tarlatzis BC, Haseltine FP, Preston SL, Soodak LK, Mattson GF, Chi AS. Luteolytic hormones are calcium-mediated, guanine nucleotide antagonists of gonadotrophin-sensitive adenylate cyclase. Adv Prostaglandin Thrombaxane Leukot Res 1985; 15:601-4.

Behrman HR, Aten RF, Ireland JJ, Milvae RA. Characteristics of an antigonadotrophic GnRH-like protein in the ovaries of diverse mammals. J Reprod Fertil Suppl 1989; 37(189):189-94.

Benhaim A, Bonnamy PJ, Papadopoulos V, Mittre H, Leymarie P. In vitro action of $PGF_{2\alpha}$ on progesterone and cAMP synthesis in small bovine luteal cells. Prostaglandins 1987; 33(2):227-39.

Bennegard B, Dennefors B, Hamberger L. Interaction between catecholamines and prostaglandin $F_{2\alpha}$ in human luteolysis. Acta Endocrinol (Copenh) 1984; 106:532-7.

Bhasin S, Heber D, Steiner B, Peterson M, Blaisch B, Campfield LA, Swerdloff RS. Hormonal effects of GnRH agonist in the human male: II. Testosterone enhances gonadotrophin suppression induced by GnRH agonist. Clin Endocrinol (Oxf) 1984; 20(2):119-28.

Bilinska B, Wojtusiak A. Effect of prostaglandins on hormonal function of cultured rat Leydig cells. Folia Histochem Cytobiol 1988; 26(2):53-9.

Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. J Biol Chem 1995; 270:18910-6.

Boie Y, Rushmore TH, Darmon GA, Grygorczyk R, Slipetz DM, Metters KM, Abramovitz M. Cloning and expression of a cDNA for the human prostanoid IP receptor. J Biol Chem 1994; 269:12173-8.

Brambaifa N, Schillinger E. Binding of prostaglandin $F_{2\alpha}$ and 20 alpha hydroxysteroid-dehydrogenase activity of immature rat ovaries throughout pseudopregnancy. Prostaglandins 1984; 14:225-234.

Bramley TA, Menzes GS, Baird DT. Specificity of gonadotrophin-releasing hormone binding sites of the human corpus luteum: comparison with receptors of rat pituitary gland. J Endocrinol 1986; 108:323-328.

Briggs MM, Lefkowitz RJ. The ß-adrenergic receptor system: a model for the transmembrane regulation of adenylate cyclase. In: Conn PM (eds.), Cellular regulation of secretion and release, Toronto, Ontario: Academic Press; 1982:23-50.

Buckler HM, Phillips SE, Kovacs GT, Burger HG, Healy DL. GnRH agonist administration in polycystic ovary syndrome. Clinical Endocrinology 1989; 31(2):151-65.

Burger HG. Neuroendocrine Control of Human Ovulation. Int J of Fertility 1981; 26(3):153-60.

Bussenot I, Azoulay-Barjonet C, Parinaud J. Modulation of steroidogenesis of cultured human granulosa-lutein cells by gonadotrophin-releasing hormone analogues. J Clin Endocrinol Metab 1993; 76:1376-1379.

Bussmann LE. Prostaglandin $F_{2\alpha}$ receptors in corpora lutea of pregnant rats and relationship with induction of 20 alpha-hydroxysteroid dehydrogenase. J Reprod Fertil 1989; 85:331-41.

Cameron IT, Baird DT. The return to ovulation following early abortion: a comparison between vacuum aspiration and prostaglandin. Acta Endocrinol (Copenh) 1988; 118:161-7.

Casper RF, Cotterell MA. The effects of adrenergic and cholinergic agents on progesterone production by human corpus luteum in vitro. Am J Obstet Gynecol 1984; 148:663-9.

Carroll RS, Corrigan AZ, Gharib SD, Vale W, Chin WW. Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. Molecular Endocrinology 1989; 3(12):1969-76.

Channing CP, Schaerf FW, Anderson LD, Tsafriri A. Ovarian follicular and luteal physiology. International Review of Physiology 1980; 22(117):117-201.

Chegini N, Ramani N, Rao CV. Morphological and biochemical characterization of small and largebovine luteal cells during pregnancy. Mol Cell Endocrinol 1984; 37(1):89-102.

Chinoy NJ, Sharma JD, Seethalakshmi L, Sanjeevan AG. Effects of prostaglandins on histophysiology of male reproductive organs and fertility in rats. Int J Fertil 1980; 25(4):267-74.

Clayton RN, Harwood JP, Catt KJ. Gonadotrophin-releasing hormone analogue binds to luteal cells and inhibits progesterone production. Nature 1979; 282:90-2.

Clayton RN, Huhtaniemi IT. Absence of gonadotrophin-releasing hormone receptors in human gonadal tissue. Nature 1982; 299:56-59.

Cockcroft S, Stutchfield J. G-proteins, the inositol lipid signalling pathway, and secretion. Philos Trans R Soc Lond B Biol Sci 1988; 320(1199):247-65 [Review].

Cohen LR, Rimon G. Prostaglandin E_2 can bimodally inhibit and stimulate the epididymal adipocyte adenylyl cyclase activity. Cell Signal 1992; 4:331-5.

Combarnous Y. Structure and structure-function relationships in gonadotrophins. Reproduction, Nutrition, Development 1988; 28(2A):211-28 [Review].

Concannon PW, Hansel W. Prostaglandin $F_{2\alpha}$ induced luteolysis, hypothermia, and abortions in beagle bitches. Prostaglandins 1977; 13:533-42.

Crumeyrolle AM, Latouche J, Laniece P, Charon Y, Tricoire H, Valentin L, Roux P, Mirambeau G, Leblanc P, Fillion G et al. "In situ" characterization of GnRH receptors: use of two radioimagers and comparison with quantitative autoradiography. J Recept Res 1994; 14:251-65.

Currie WD, Li W, Baimbridge KG, Ho Yuen B, Leung PCK. Cytosolic free calcium increased by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), gonadotrophin-releasing hormone, and angiotensin II in rat granulosa cells and $PGF_{2\alpha}$ in human granulosa cells. Endocrinology 1992; 130:1837-43.

Davis JS, West LA, Farese RV. Gonadotrophin-releasing hormone rapidly alters polyphosphoinositide metabolism in rat granulosa cells. Biochem Biophys Res Commun 1984; 122(3):1289-95.

Davis JS, West LA, Farese RV. Gonadotrophin-releasing hormone (GnRH) rapidly stimulates the formation of inositol phosphates and diacyglycerol in rat granulosa cells: further evidence for the involvement of Ca^{2+} and protein kinase C in the action of GnRH. Endocrinology 1986;. 118:2561-71.

Davis JS, West LA, Farese RV. Gonadotrophin-releasing hormone (GnRH) rapidly alters polyphosphoinositide metabolism in rat granulosa cells. Biochem Biophys Res Commun 1984; 122(3):1289-95.

Davis JS, Tedesco TA, West LA, Maroulis GB, Weakland LL. Effects of human chorionic gonadotrophin, prostaglandin $F_{2\alpha}$ and protein kinase C activators on the cyclic AMP and inositol phosphate second messenger systems in cultured human granulosa-luteal cells. Mol Cell Endocrinol 1989; 65:187-93.

Davis PD, Hill CH, Lawton G, Nixon JS, Wilkinson SE, Hurst SA, Keech E, Turner SE. Inhibitors of protein kinase C. 1. 2,3-Bisarylmaleimides. J Med Chem 1992; 35:177-84.

Dennis EA. Phospholipases. In: Boyer PD (ed.), The enzymes, vol. 16, 3rd ed., Academic Press; 1983:307-353.

DePaolo LV, Mercado M, Guo Y, Ling N. Increased follistatin (activin-binding protein) gene expression in rat anterior pituitary tissue after ovariectomy may be mediated by pituitary activin. Endocrinology 1993; 132(5):2221-8.

Donham RS, Champney TH, Kerner T, and Stetson MH. Temporary anestrus induced by injection of luteinizing hormone-releasing hormone in hamsters. Biology of Reproduction 1993; 48(5):1135-40.

Dong K-W, Yu K-L, Roberts JL. Identification of a major up-stream transcription start site for the human progonadotrophin-releasing hormone gene used in reproductive tissues and cell lines. Mol Endocrinol 7:1654-1666.

Dorflinger LJ, Luborsky JL, Gore SD, Behrman HR. Inhibitory characteristics of prostaglandin $F_{2\alpha}$ in the rat luteal cell, Mol Cell Endocrinol 1983; 33(2-3):225-41.

Dorflinger LJ, Albert PJ, Williams AT, Behrman HR. Calcium is an inhibitor of luteinizing hormone-sensitive adenylate cyclase in the luteal cell. Endocrinology 1984; 114(4):1208-15.

Dufour S., Lopez E, Le MF, Le BN, Baloche S, Fontaine YA. Stimulation of gonadotrophin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. General & Comparative Endocrinology 1988; 70(1):20-30.

Endo T, Yamamoto H, Tanaka S. Effect of prostaglandins(PGs) on progesterone production by human cultured luteal cells and their ability of PGs production. Nippon Naibunpi Gakkai Zasshi 1988; 64:687-97.

Endo T, Watanabe H, Yamamoto H, Tanaka S. and Hashimoto M. Prostaglandin $F_{2\alpha}$- and phorbol 12-myristate-13-acetate-stimulated progesterone production by cultured human luteal cells in the mid-luteal phase: prostaglandin $F_{2\alpha}$ increases cytosolic Ca^{2+} and inositol phosphates. J Endocrinol 1992; 133(3):451-8.

Fan NC, Jeung EB, Peng C, Olofsson JI, Krisinger J, Leung PCK. The human gonadotrophin-releasing hormone (GnRH) receptor gene: cloning, genomic organization and chromosomal assignment. Mol Cell Endocrinol 1994; 103(1-3):R1-6.

Farin PW, Estill CT. Infertility due to abnormalities of the ovaries in cattle. Vet Clin North Am Food Anim Pract 1993; 9:291-308 [Review].

Fields MJ, Barros CM, Watkins WB, Fields PA. Characterization of large luteal cells and their secretory granulesduring the estrous cycle of the cow. Biol Reprod 1992; 46(4):535-45.

Fields MJ, Dubois W, Fields PA. Dynamic features of luteal secretory granules: ultrastructuralchanges during the course of pregnancy in the cow. Endocrinology 1985; 117(4):1675-82.

Fielding CJ, Fielding PE. Metabolism of Cholesterol and Lipoproteins. In: Vance DE, Vance JE (eds.), Biochemistry of Lipids and Membranes, Menlo Park, California: The Benjamin/Cummings Publishing Co. Inc.; 1985:404-424.

Filicori M., Bolelli G., Franceschetti F, Lafisca S. The ultradian pulsatile release of gonadotrophins in normal female subjects. Acta Europaea Fertilitatis 1979; 10(1):29-33.

Findlay JK. An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. Biology of Reproduction 1993; 48(1):15-23.

Findlay JK, Sai X, Shukovski L. Role of inhibin-related peptides as intragonadal regulators. Reproduction, Fertility, & Development 1990; 2(3):205-18.

Fisher LD, van Belle G. Biostatistics: a methodology for the health sciences. John Wiley & Sons, Inc. Toronto, 1993.

Fleming N, Sliwinski LE, Burke DN. G regulatory proteins and muscarinic receptor signal transduction in mucous acini of rat submandibular gland. Life Sci 1989; 44(15):1027-35.

Fohr KJ, Mayerhofer A, Sterzik K, Rudolf M, Rosenbusch B, Gratzl M. Concerted action of human chorionic gonadotrophin and norepinephrine on intracellular-free calcium in human granulosa-lutein cells: evidence for the presence of a functional alpha-adrenergic receptor. J Clin Endocrinol Metab 1993; 76:367-73

Franz W3. Basic review: endocrinology of the normal menstrual cycle. Prim Care 1988; 15:607-16 [Review].

Fraser HM. GnRH analogues for contraception. British Medical Bulletin 1993, 49(1):62-72 [Review].

Fuchs AR, Smitasiri Y, Chantharaksri U. The effect of indomethacin on uterine contractility and luteal regression in pregnant rats at term. J Reprod Fertil 1976; 48:331-40.

Fuchs AR. Oxytocin and ovarian function. J Reprod Fertil Suppl 1988; 36:39-47.

Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M, Metters KM. Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. J Biol Chem 1993; 268:26767-72.

Funston RN, Seidel GJ. Gonadotropin-releasing hormone increases cleavage rates of bovine oocytes fertilized in vitro. Biol Reprod 1995; 53:541-5.

Genuth SM. The endocrine system. In: Berne RM, Levy MN (eds.), Physiology, 2nd ed., Toronto, Ontario: The CV Mosby Co.; 1988; 983-1024.

Girsh E, Greber Y, Meidan R. Luteotrophic and luteolytic interactions between bovine small and large luteal-like cells and endothelial cells. Biol Reprod 1995; 52(4):954-62.

Gitay-Goren H, Brandes JM, Bar-Ami S. Altered steroidogenic pattern of human granulosa-lutein cells in relation to cumulus cell culture morphology. J Steroid Biochem 1990; 36(5):457-64.

Goebelsmann U. Protein and steroid hormones in pregnancy. J Reprod Med 1979; 23:166-77 [Review].

Gonen Y, Dirnfeld M, Goldman S, Koifman M, Abramovici H. The use of long-acting gonadotrophin-releasing hormone agonist (GnRH-a; decapeptyl) and gonadotrophins versus short-acting GnRH-a (buserelin) and gonadotrophins before and during ovarian stimulation for in vitro fertilization (IVF). Journal of in Vitro Fertilization & Embryo Transfer 1991; 8(5):254-9.

Goubau S, Bond CT, Adelman JP, Misra V, Hynes MF, Schultz GA, Murphy BD. Partial characterization of the gonadotrophin-releasing hormone (GnRH) gene transcript in the rat ovary. Endocrinology 1992; 130:3098-3100.

Granner DK. Hormones of the gonads. In: Murray RK, Granner DK, Mayes PA, Rodwell VW (eds.), Harper's Biochemistry, 21st ed., San Mateo, California, Appleton & Lange; 1988:536-546.

Gray CJ. Glycoprotein gonadotrophins. Structure and synthesis. [Review]. Acta Endocrinologica, Supplement 1988; 288(20):20-7.

Gregoraszczuk E, Krzysztofowicz E. The corpus luteum of the pig. Scanning electron microscopic study of surface features at different times of incubation. Acta Biol Hung 1989; 40(1-2):145-56.

Grinwich DL, Ham EA, Hichens M, Behrman HR. Binding of human chorionic gonadotrophin and response of cyclic nucleotides to luteinizing hormone in luteal tissue from rats treated with prostaglandin $F_{2\alpha}$. Endocrinology 1976; 98:146-50.

Grimm LG, Yarnold PR. Reading and understanding multivariate statistics. American Psychological Association, Washington DC, 1996.

Hall JE, Whitcomb RW, Rivier JE, Vale WW, Crowley WJ. Differential regulation of luteinizing hormone, follicle-stimulating hormone, and free alpha-subunit secretion from the gonadotrope by gonadotrophin-releasing hormone (GnRH): evidence from the use of two GnRH antagonists. Journal of Clinical Endocrinology & Metabolism 1990; 70(2):328-35.

Hanzen C. Prostaglandins and the physiology of human and animal reproduction. J Gynecol Obstet Biol Reprod (Paris) 1984; 13:351-61.

Haour F, Lang B. Role of hormonal receptors in the regulation of the corpus luteum (author's transl). [French]. Semaine Des Hopitaux De Paris 1978; 54(33-36):1063-70.

Hargrove JL, Seeley RR, Ellis LC. Rabbit testicular contractions: bimodal interaction of prostaglandin E1 with other agonists. Am J Physiol 1975; 228:810-4.

Harris KH, Murphy BD. Luteolysis in the hamster: abrogation by gonadotrophin and prolactin pretreatment. Prostaglandins 1981; 21(2):177-87.

Harris KH, Murphy BD. Luteolysis in the hamster: abrogation by gonadotrophin and prolactin pretreatment. Prostaglandins 1981; 21(2):177-87.

Harvey HA, Lipton A, Max DT, Pearlman HG, Diaz PR, de, la, Garza J. Medical castration produced by the GnRH analogue leuprolide to treat metastatic breast cancer. Journal of Clinical Oncology 1985; 3(8):1068-72.

Hawes BE, Waters SB, Janovick JA, Bleasdale JE, Conn PM. Gonadotrophin-releasing hormone-stimulated intracellular Ca2+ fluctuations and luteinizing hormone release can be uncoupled from inositol phosphate production. Endocrinology 1992; 130(6):3475-83.

Hawes BE, Barnes S, Conn PM. Cholera toxin and pertussis toxin provoke differential effects on luteinizing hormone release, inositol phosphate production, and gonadotrophin-releasing hormone (GnRH) receptor binding in the gonadotrope: evidence for multiple guanyl nucleotide binding proteins in GnRH action. Endocrinology 1993; 132:2124-30.

Hillensjo T, LeMaire WJ, Clark MR, Ahren K. Effect of gonadotrophin-releasing hormone (GnRH) and GnRH agonists upon accumulation of progesterone, cAMP and prostaglandin in isolated preovulatory rat follicles. Acta Endocrinol (Copenh) 1982; 101(4):603-10.

Hillensjo T, Sjogren A, Strander B, Nilsson L, Wikland M, Hamberger L, Roos P. Effect of gonadotrophins on progesterone secretion by cultured granulosa cells obtained from human preovulatory follicles, Acta Endocrinol (Copenh) 1985; 110(3):401-7.

Hillier SG, Zeleznik AJ, Knazek RA, Ross GT. Hormonal regulation of preovulatory follicle maturation in the rat. J Reprod Fertil 1980; 60:219-29.

Hillier SG, Miro F. Inhibin, activin, and follistatin. Potential roles in ovarian physiology. Annals of the New York Academy of Sciences 1993; 687(29):29-38.

Hsueh AJW, Schaeffer JM. Gonadotrophin-releasing hormone as a paracrine hormone and neurotransmitter in extra-pituitary sites. J Steroid Biochem 1985; 23:757-764.

Hsueh MJ, Adashi EY, Tucker E, Valk C, Ling N C. Relative potencies of gonadotrophin-releasing hormone agonists and antagonists on ovarian and pituitary functions. Endocrinology 1983; 112(2):689-95.

Igarashi M. Mini review on inhibins and activins. [Japanese]. Nippon Naibunpi Gakkai Zasshi Folia Endocrinologica Japonica 1992; 68(2):71-80.

Inaudi P, Reymond MJ, Rey F, Genazzani AD, Lemarchand BT. Pulsatile secretion of gonadotrophins and prolactin during the follicular and luteal phases of the menstrual cycle: analysis of instantaneous secretion rate and secretory concomitance. Fertility & Sterility 1992; 58(1):51-9.

Ireland JJ, Aten RF, Behrman HR. GnRH-like proteins in cows: concentrations during corpora lutea development and selective localization in granulosal cells. Biol Reprod 1988; 38(3):544-50.

Jalkanen J, Ritvos O, Huhtaniemi I, Stenman UH, Laatikainen T, Ranta T. Phorbol ester stimulates human granulosa-luteal cell cyclic adenosine 3', 5'-monophosphate and progesterone production. Mol Cell Endocrinol 1987; 51:273-6.

Janne OA. Progesterone action in mammalian uterus. Acta Obstet Gynecol Scand Suppl 1981; 101:11-6 [Review].

Jansen RP. Ultrastructure and histochemistry of acid mucus glycoproteins in the estrous mammal oviduct. Microsc Res Tech 1995; 32:24-49 [Review].

Jones PB, Valk CA, Hsueh AJ. Regulation of progestin biosynthetic enzymes in cultured rat granulosa cells: effects of prolactin, beta 2-adrenergic agonist, human chorionic gonadotrophin and gonadotrophin-releasing hormone. Biol Reprod 1983; 29(3):572-85.

Joshi D, Lekhtman I, Billiar RB, Miller M. Gonadotrophin-releasing hormone induced luteinizing hormone responses in young and old female C57BL/6J mice. Proceedings of the Society for Experimental Biology & Medicine 1993; 204(2): 191-4,.

Kaiser UB, Lee BL, Carroll RS, Unabia G, Chin WW, Childs GV. Follistatin gene expression in the pituitary: localization in gonadotropes and folliculostellate cells in diestrous rats. Endocrinology 1992; 130(5):3048-56.

Kawai Y, Clark MR. Phorbol ester regulation of rat granulosa cell prostaglandin and progesterone accumulation. Endocrinology 1985; 116(6):2320-6.

Kakar SS, Musgrove LC, Devor DC, Sellers JC, Neill JD. Cloning, sequencing, and expression of human gonadotrophin-releasing hormone (GnRH) receptor. Biochem Biophys Res Commun 1992; 189:289-295.

Kakar SS, Grantham K, Musgrove LC, Devor D, Sellers JC, Neill JD. Rat gonadotrophin-releasing hormone (GnRH) receptor: tissue expression and hormonal regulation of its mRNA. Mol Cell Endocrinol 1994; 101:151-157.

Kawai Y, Clark MR. Mechanisms of action of gonadotrophin-releasing hormone on rat granulosa cells. Endocr Res 1986; 12(3):195-209.

Kammerman S, Ross J. Increase in numbers of gonadotrophin receptors on granulosa cells during follicle maturation. J Clin Endocrinol Metab 1975; 41:546-50.

Khan DF, Huang JC, Dawood MY. Effect of human chorionic gonadotrophin and prostaglandin $F_{2\alpha}$ on progesterone production by human luteal cells. J Steroid Biochem 1989; 33:941-7.

Kiesel L, Bertges K, Rabe T, Runnebaum B. Gonadotrophin-releasing hormone enhances polyphosphoinositide hydrolysis in rat pituitary cells. Biochem Biophys Res Commun 1986; 134(2):861-7.

King JA, Mehl AE, Tyndale BC, Hinds L, Millar RP. A second form of gonadotrophin-releasing hormone (GnRH), with chicken GnRH II-like properties, occurs together with mammalian GnRH in marsupial brains. Endocrinology 1989; 125(5):2244-52.

King JA, Hinds LA, Mehl AE, Saunders NR, Millar RP. Chicken GnRH II occurs together with mammalian GnRH in a South American species of marsupial (Monodelphis domestica). Peptides 1990; 11(3):521-5.

Korda AR, Shutt DA, Smith ID, Shearman RP, Lyneham RC. Assessment of possible luteolytic effect on intra-ovarian injection of prostaglandin $F_{2\alpha}$ in the human. Prostaglandins 1975; 9:443-9.

Laatikainen TJ. Corticotropin-releasing hormone and opioid peptides in reproduction and stress. Annals of Medicine 1991; 23(5):489-96 [Review].

Lahav M, Rennert H, Sabag K, Barzilai D. Calmodulin inhibitors and 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate do not prevent the inhibitory effect of prostaglandin $F_{2\alpha}$ on cyclic AMP production in isolated rat corpora lutea. J Endocrinol 1987; 113:205-12.

Lahav M, West LA, Davis JS. Effects of prostaglandin $F_{2\alpha}$ and a gonadotrophin-releasing hormone agonist on inositol phospholipid metabolism in isolated rat corpora lutea of various ages. Endocrinology 1988; 123(2):1044-52.

Lahav M, Davis JS, Rennert H. Mechanism of the luteolytic action of prostaglandin $F_{-2\alpha}$ in the rat. J Reprod Fertil Suppl 1989; 37(233):233-40.

Lake S, Gullberg H, Wahlqvist J, Sjogren AM, Kinhult A, Lind P, Hellstrom LE, Stjernschantz J. Cloning of the rat and human prostaglandin $F_{2\alpha}$ receptors and the expression of the rat prostaglandin $F_{2\alpha}$ receptor. Febs Lett 1994; 355:317-25.

Langlois D, Hinsch KD, Saez JM, Begeot M. Stimulatory effect of insulin and insulin-like growth factor I on Gi proteins and angiotensin-II-induced phosphoinositide breakdown in cultured bovine adrenal cells. Endocrinology 1990; 126(4):1867-72.

Latouche J, Crumeyrolle AM, Jordan D, Kopp N, Augendre FB, Ceddard L, Haour F. GnRH receptors in human granulosa cells: anatomical localization and characterization by autoradiographic study. Endocrinology 1989; 125(3):1739-41.

Lau IF, Hoogasian J, Wong SK,Saksena SK. Effects of prostaglandin $F_{2\alpha}$ and an antiprogestational steroid, RMI 12,936, on rat pregnancy. Prostaglandins Med 1980; 4:121-32.

Lee BL, Unabia G, Childs G. Expression of follistatin mRNA by somatotropes and mammotropes early in the rat estrous cycle. Journal of Histochemistry & Cytochemistry 1993; 41(7):955-60.

Leung PCK, Armstrong DT. Stimulatory action of follicle-stimulating hormone and androgens on the responsiveness of rat granulosa cells to gonadotrophins *in vitro*. Endocrinology 1979; 104:1124-1129.

Leung PCK. Mechanisms of gonadotrophin-releasing hormone and prostaglandin action on luteal cells. Can J Physiol Pharmacol 1985; 63(3):249-56.

Leung PCK, Steele GL. Intracellular signaling in the gonads. Endocr Rev 1992; 13(3):476-98 [Review].

Lewis AE, Edwards A. Biostatistics. 1984; 2nd ed. Van Nostrand Reinhold. New York.

Li W, Khorasheh S, Ho Yuen B, Ling N, Leung PCK. Stimulation of progesterone secretion by recombinant follistatin-288 in human granulosa cells. Endocrinology 1993; 132:1750-1756.

Ling N, DePaolo LV, Bicsak TA, Shimasaki S. Novel ovarian regulatory peptides: inhibin, activin, and follistatin. Clinical Obstetrics & Gynecology 1990; 33(3):690-702.

Lopez BA, Bellinger J, Marshall JM, Phaneuf S, Europe FG, Asboth G, Barlow DH. G protein expression and second messenger formation in human granulosa cells. J Reprod Fertil 1995; 104:77-83.

Luborsky JL, Slater WT, Behrman HR. Luteinizing hormone (LH) receptor aggregation: modification of ferritin-LH binding and aggregation by prostaglandin $F_{2\alpha}$ and ferritin-LH. Endocrinology 1984; 115(6):2217-26.

Ma F, Leung PCK. Luteinizing hormone-releasing hormone enhances polyphosphoinositide breakdown in rat granulosa cells. Biochemical & Biophysical Research Communications 1985; 130(3):1201-8.

Magnaldo I, Pouyssegur J, Paris S. Thrombin exerts a dual effect on stimulated adenylate cyclase in hamster fibroblasts, an inhibition via a GTP-binding protein and a potentiation via activation of protein kinase C. Biochem J 1988; 253(3):711-9.

Manaugh LC, Novy MJ. Effects of indomethacin on corpus luteum function and pregnancy in rhesus monkeys. Fertil Steril 1976; 27:588-98.

Mahesh VB. The dynamic interaction between steroids and gonadotropins in the mammalian ovulatory cycle. Neurosci Biobehav Rev 1985; 9:245-60 [Review].

Martin KA, Liptrap RM. Half-life of 13,14dihydro-15-keto prostaglandin $F_{2\alpha}$ in peripheral plasma of the pig. Res Vet Sci 1981; 31(3):387-9.

Martin K, Santoro N, Hall J, Filicori M, Wierman M, Crowley WJ. Clinical review 15: Management of ovulatory disorders with pulsatile gonadotrophin-releasing hormone. Journal of Clinical Endocrinology & Metabolism 1990; 71(5):1081A-1081G [Review].

Martin TF, Lewis JE, Kowalchyk JA. Phospholipase C-beta 1 is regulated by a pertussis toxin-insensitive G-protein. Biochem J 1991; 280: 753-60.

Massicotte J, Lachance R, Labrie F. Modulation of cyclic AMP formation and progesterone secretion by human chorionic gonadotrophin, epinephrine, buserelin and prostaglandins in normal or human chorionic gonadotrophin desensitized rat immature luteal cells in monolayer culture. J Steroid Biochem 1984;. 21:745-54.

Mates G, Daniel M, Walker C. Factors affecting the reproducibility of a spectrofluoroimetric assay for the enumeration of human venous endothelium in culture. Cell Biol Int Rep 1986; 10:641-8.

Mauvais JP, Kuttenn F, Gompel A. Estradiol/progesterone interaction in normal and pathologic breast cells. Ann N Y Acad Sci 1986; 464:152-67 [Review].

Mauvais JP, Kuttenn F, Gompel A. Antiestrogen action of progesterone in breast tissue. Horm Res 1987; 28:212-8 [Review].

Mauvais JP, Kuttenn F,Gompel A. Antiestrogen action of progesterone in breast tissue. Breast Cancer Res Treat 1986; 8:179-88 [Review].

May JV, Schomberg DW. Developmental coordination of luteinizing hormone/human chorionic gonadotrophin (hCG) receptors and acute hCG responsiveness in cultured and freshly harvested porcine granulosa cells. Endocrinology 1984; 114:153-63.

Mayes PA. Lipids of Physiologic Significance. In: Murray RK, Granner DK, Mayes PA, Rodwell VW (eds.), Harper's Biochemistry, 21st ed., San Mateo, California, Appleton & Lange; 1988:130-137.

McArdle CA, Poch A, Schomerus E, Kratzmeier M. Pituitary adenylate cyclase-activating polypeptide effects in pituitary cells: modulation by gonadotrophin-releasing hormone in alpha T3-1 cells. Endocrinology 1994; 134(6):2599-605.

McCann TJ, Flint AP. Use of pertussis toxin to investigate the mechanism of action of prostaglandin $F_{2\alpha}$ on the corpus luteum in sheep. J Mol Endocrinol 1993; 10(1):79-85.

McCarty KJ, Lubahn DB, McCarty KS. Oestrogen and progesterone receptors: physiological and pathological considerations. Clin Endocrinol Metab 1983; 12:133-54.

McCarthy DM. Mechanisms of mucosal injury and healing: the role of non-steroidal anti-inflammatory drugs. Scand J Gastroenterol Suppl 1995; 208:24-9.

McNatty KP, Smith DM, Makris A, Osathanondh R, Ryan KJ. The microenvironment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the population of granulosa cells, and the status of the oocyte in vivo and in vitro. J Clin Endocrinol Metab 1979; 49:851-60.

McNatty KP, Makris A, De GC, Osathanondh R, Ryan KJ. The production of progesterone, androgens and oestrogens by human granulosa cells in vitro and in vivo. J Steroid Biochem 1979; 11:775-9.

Meidan R, Girsh E, Blum O, Aberdam E. In vitro differentiation of bovine theca and granulosa cells intosmall and large luteal-like cells: morphological and functional characteristics. Biol Reprod 1990; 43(6):913-21.

Meldrum DR, Chang RJ, Lu J, Vale W, Rivier J, Judd HL. *Medical oophorectomy* using a long-acting GNRH agonist–a possible new approach to the treatment of endometriosis. Journal of Clinical Endocrinology & Metabolism 1982; 54(5):1081-3.

Mercado M, Shimasaki S, Ling N, DePaolo L. Effects of estrous cycle stage and pregnancy on follistatin gene expression and immunoreactivity in rat reproductive tissues: progesterone is implicated in regulating uterine gene expression. Endocrinology 132(4):1774-81, 1993.

Michael AE, Abayasekara DR, Webley GE. The luteotrophic actions of prostaglandins E_2 and $F_{2\alpha}$ on dispersed marmoset luteal cells are differentially mediated via cyclic AMP and protein kinase C. J Endocrinol 1993; 138:291-8.

Michael AE, Webley GE. Roles of cyclic AMP and inositol phosphates in the luteolytic action of cloprostenol, a prostaglandin $F_{2\alpha}$ analogue, in marmoset monkeys (Callithrix jacchus). J Reprod Fertil 1993; 97:425-31.

Michael AE, Webley GE. Prostaglandin $F_{2\alpha}$ stimulates cAMP phosphodiesterase via protein kinase C in cultured human granulosa cells. Mol Cell Endocrinol 1991a; 82:207-14.

Michael AE, Webley GE. Prior exposure to gonadotrophins prevents the subsequent antigonadotrophic actions of cloprostenol by a cyclic AMP-dependent mechanism in cultured human granulosa cells. J Endocrinol 1991b; 131:319-25.

Michel U, Albiston A, Findlay JK. Rat follistatin: gonadal and extragonadal expression and evidence for alternative splicing. Biochemical & Biophysical Research Communications 1990; 173(1):401-7.

Michel U, McMaster JW, Findlay JK. Regulation of steady-state follistatin mRNA levels in rat granulosa cells in vitro. Journal of Molecular Endocrinology 1992; 9(2):147-56.

Minaretzis D, Jakubowski M, Mortola JF, Pavlou SN. Gonadotrophin-releasing hormone receptor gene expression in human ovary and granulosa-lutein cells. J Clin Endocrinol Metab 1995; 80(2):430-4.

Mitsuhashi N. Studies on the mechanism and the significance of prostaglandin biosynthesis by the ovary–ovulation block by the indomethacin and incubation of the follicle. Acta Obstet Gynaecol Jpn 1981; 33:479-88.

Molcho J, Zakut H, Naor Z. Gonadotrophin-releasing hormone stimulates phosphatidylinositol labeling and prostaglandin E production in Leydig cells. Endocrinology 1984; 114(3):1048-50

Moon YS, Duleba AJ. Comparative studies of androgen metabolism in theca and granulosa cells of human follicles in vitro. Steroids 1982; 39:419-30.

Moon YS. The role of gonadotropins and testosterone in progesterone production by human ovarian granulosa cells. Mol Cell Endocrinol 1981; 23:115-22.

Moon YS, Tsang BK, Simpson C, Armstrong DT. 17 beta-Estradiol biosynthesis in cultured granulosa and thecal cells of human ovarian follicles: stimulation by follicle-stimulating hormone. J Clin Endocrinol Metab 1978; 47:263-7.

Moon YS, Duleba AJ, Kim KS, Ho Yuen B. Effects of prostaglandins E_2 and $F_{2\alpha}$ on progesterone metabolism by rat granulosa cells. Biochem Biophys Res Commun 1986; 135:764-9.

Nakatani A, Shimasaki S, Depaolo LV, Erickson GF, Ling N. Cyclic changes in follistatin messenger ribonucleic acid and its protein in the rat ovary during the estrous cycle. Endocrinology 1991; 129(2):603-11.

Nillius SJ, Gemzell C, Johansson ED, Wide L. Monitoring treatment with human gonadotrophins or the synthetic decapeptide LH-releasing hormone. pp. 753-75, In: Crosignani Pg, James Vh, ed. Recent progress in reproductive endocrinology. 1974; London, Academic Press, 4.

Nebert DW, Gonzales FJ. P450 genes: structure, evolution, and regulation. Ann Rev Biochem 1987; 56:945-993.

Netiv E, Liscovitch M, Naor Z. Delayed activation of phospholipase D by gonadotrophin-releasing hormone in a clonal pituitary gonadotrope cell line (alpha T3-1). Febs Lett 1991; 295(1-3):107-9.

Nelson SE, McLean MP, Jayatilak PG, Gibori G. Isolation, characterization, and culture of cell subpopulationsforming the pregnant rat corpus luteum. Endocrinology 1992; 130(2):954-66.

Nillius SJ. Gonadotrophin-releasing hormone agonists for new approaches to contraception in man. Wien Klin Wochenschr 1985; 97(23):865-73 [Review].

Ng sy, Gunning P, Eddy R, Ponte P, Leavitt J, Shows T, Kedes L. Evoloution of the functional human ß-actin gene and its multipseudogene family: Conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol Cell Biol 1985; 5:2720-2732.

O'Dowd BF, Hnatowich M, Regan JW, Leader WM, Caron MG, Lefkowitz RJ, Site-directed mutagenesis of the cytoplasmic domains of the human beta 2-adrenergic receptor. Localization of regions involved in G protein-receptor coupling, J Biol Chem 1988; 263:15985-92.

Ohno T, Imai A, Furui T, Takahashi K, Tamaya T. Presence of gonadotrophin-releasing hormone and its messenger ribonucleic acid in human ovarian epithelial carcinoma. Am J Obstet Gynecol 1993; 169:605-610.

Oikawa M, Dargan C, Ny T, Hsueh AJ. Expression of gonadotrophin-releasing hormone and prothymosin-alpha messenger ribonucleic acid in the ovary. Endocrinology 1990; 127(5):2350-6.

Olofsson JI, Conti CC, Leung C, Krisinger J, Leung PCK. Tissue-specific regulation of gonadotrophin-releasing hormone (GnRH) receptor gene expression during the periovulatory period. Endocr J 1994; 2:471-476.

Olsson J-H, Akesson I, Hillensjo T. Effects of a gonadotrophin-releasing hormone agonist on progesterone formation in cultured human granulosa cells. Acta Endocrinol (Copenh) 1990; 122:427-431.

Orlicky DJ, Williams SC. Immunohistochemical localization of $PGF_{2\alpha}$ receptor in the mouse testis. Prostaglandins Leukot Essent Fatty Acids 1992; 47(4):247-52.

Parinaud J, Vieitez G, Beaur A, Pontonnier G, Boureau E. Effect of a luteinizing hormone-releasing hormone agonist (buserelin) on steroidogenesis of cultured human preovulatory granulosa cells. Fertil Steril 1988; 50:597-602.

Patwardhan VV, Lanthier A. Concentration of prostaglandins PGE and PGF, estrone, estradiol, and progesterone in human corpora lutea. Prostaglandins 1980; 20:963-9.

Patwardhan VV, Lanthier A. Prostaglandins PGE and PGF in human ovarian follicles: endogenous contents and in vitro formation by theca and granulosa cells. Acta Endocrinol (Copenh) 1981; 97:543-50.

Patwardhan VV, Lanthier A. Luteal phase variations in endogenous concentrations of prostaglandins PGE and PGF and in the capacity for their in vitro formation in the human corpus luteum. Prostaglandins 1985; 30(1):91-8.

Pellicer A, Tarin JJ, Miro F, Sampaio M, De-los-Santos, Mj, Remohi J. The use of gonadotrophin-releasing-hormone analogues (GnRHa), in in-vitro fertilization: some clinical and experimental investigations of a direct effect on the human ovary. Human Reproduction 1992. 1(39):39-47.

Peng C, Fan NC, Ligier M, Väänänen JE, Leung PCK. Expression and regulation of gonadotrophin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. Endocrinology 1994; 135(5):1740-6.

Pepperell JR, Preston SL, Behrman HR. The antigonadotropic action of prostaglandin $F_{2\alpha}$ is not mediated by elevated cytosolic calcium levels in rat luteal cells. Endocrinology 1989; 125:144-51.

Plunkett ER, Moon YS, Zamecnik J, Armstrong DT. Preliminary evidence of a role for prostaglandin F in human follicular function. Am J Obstet Gynecol 1975; 123:391-7.

Popkin R, Bramley TA, Currie A, Shaw RW, Baird DT, Fraser HM. Specific binding of luteinizing hormone releasing hormone to human luteal tissue. Biochem Biophys Res Commun 1983; 114:750-756.

Porkess. The Harper Collins dictionary of statistics. Harper Perennial. New York, 1991.

Puschel GP, Kirchner C, Schroder A, Jungermann K. Glycogenolytic and antiglycogenolytic prostaglandin E_2 actions in rat hepatocytes are mediated via different signalling pathways. Eur J Biochem 1993; 218:1083-9.

Quirk SM, Cowan RG, Joshi SG, Henrickson KP. Fas antigen-mediated apoptosis in human granulosa/luteal cells. Biol Reprod 1995; 52(2):279-87.

Rajkumar K, Ganguli S, Menon KM, Mead RA, Murphy BD. Studies on the mechanism of action of prostaglandin $F_{2\alpha}$ induced luteolysis in rats. Prostaglandins 1988; 36(4):547-64.

Rasmussen DD,Yen SS. Progesterone and 20 alpha-hydroxyprogesterone stimulate the in vitro release of GnRH by the isolated mediobasal hypothalamus. Life Sci 1983; 32:1523-30.

Retamales I, Carrasco I, Troncoso JL, Las Heras J, Devoto L, Vega M. Morpho-functional study of human luteal cell subpopulations. Hum Reprod 1994; 9(4):591-6.

Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Alliston T, Sirois J. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes. Recent Prog Horm Res 1995; 50:223-54 [Review].

Richardson MC, Masson GM. Progesterone production by dispersed cells from human corpus luteum: stimulation by gonadotrophins and prostaglandin $F_{2\alpha}$; lack of response to adrenaline and isoprenaline. J Endocrinol 1980; 87:247-54.

Ristimaki A, Jaatinen R and Ritvos O. Regulation of prostaglandin $F_{2\alpha}$ receptor expression in cultured human granulosa-luteal cells. Endocrinology 1997; 138:191-195.

Rodway MR, Ho yuen B, Leung PCK. Inhibition of aromatase activity by 8-Br-cAMP in cultured first trimester human trophoblast. Am J Obst Gynecol. 1990; 163:1546-1551.

Rommler A, Viebahn C, Schwartz U, Hammerstein J. Short-term regulation of LH and FSH secretion in cyclic women. III. Effects of varying doses of two consecutive LH-RH injections on pituitary and ovarian response. Acta Endocrinologica 1979; 90(3):394-402.

Roth TL, Munson L, Swanson WF, Wildt DE. Histological characteristics of the uterine endometrium and corpusluteum during early embryogenesis and the relationship to embryonicmortality in the domestic cat. Biol Reprod 1995; 53(5):1012-21.

Roth GJ, Siok CJ. Acetylation of the NH2-terminal serine of prostaglandin synthetase by aspirin. J Biol Chem 1978; 253:3782-4.

Rotsztejn WH, Charli JL, Pattou E, Epelbaum J, Kordon C. In vitro release of luteinizing hormone-releasing hormone (LHRH) from rat mediobasal hypothalamus: effects of potassium, calcium and dopamine. Endocrinology 1976; 99(6):1663-6.

Ryan RJ, Keutmann HT, Charlesworth MC, McCormick DJ, Milius RP, Calvo FO, Vutyavanich T. Structure-function relationships of gonadotrophins. Recent Progress in Hormone Research 1987; 43(383):383-429 [Review].

Sano N, Shichi H. Bimodal regulation of adenylate cyclase by prostaglandin E2 receptors in porcine ciliary epithelium. Prostaglandins Leukot Essent Fatty Acids 1993; 49:765-9.

Satoh K, Yasumizu T, Kawai Y, Ozaki A, Wu T, Kinoshita K, Sakamoto S. In vitro production of prostaglandins E, F, and 6-keto prostaglandin $F_{1\alpha}$ by human pregnant uterus, decidua and amnion. Prostaglandins Med 1981; 6:359-68.

Sawyer CH. First Geoffrey Harris Memorial lecture. Some recent developments in brain-pituitary-ovarian physiology. Neuroendocrinology 1975; 17(2):97-124 [Review].

Schroepfer GJ Jr. Sterol biosynthesis. Ann Rev Biochem 1982; 51:555-585.

Schuiling GA, Gnodde HP. Site of origin of the pulsatile secretion of luteinizing hormone in long-term ovariectomized rats. Journal of Endocrinology 1976; 70(1):97-104.

Schwartzman ML, Falck JR, Yadagiri P, Escalante B. Metabolism of 20-hydroxeicosatetraenoic acid by cyclooxygenase. Formation and identification of novel endothelium-dependent vasoconstrictor metabolites. J Biol Chem 1989; 264(20):11658-62.

Segars JH, Hill GA, Bryan SH, Herbert C3, Osteen KG, Rogers BJ, Wentz AC. The use of gonadotrophin-releasing hormone agonist (GnRHa) in good responders undergoing repeat in vitro fertilization/embryo transfer (IVF/ET). Journal of in Vitro Fertilization & Embryo Transfer 1990; 7(6):327-31.

Shimasaki S, Koga M, Buscaglia ML, Simmons DM, Bicsak TA, Ling N. Follistatin gene expression in the ovary and extragonadal tissues. Molecular Endocrinology 1989; 3(4):651-9.

Shinohara O, Knecht M, Catt KJ. Inhibition of gonadotrophin-induced granulosa cell differentiation by activation of protein kinase C. Proc Natl Acad Sci U S A 1985; 82(24):8518-22.

Shimonaka M, Inouye S, Shimasaki S, Ling N. Follistatin binds to both activin and inhibin through the common subunit. Endocrinology 1991; 128(6):3313-5.

Siler KT, Khodr GS, Valenzuela G, Harper MJ, Rhode J. GnRH effects on placental hormones during gestation. III. Prostaglandin E, prostaglandin F, and 13,14-dihydro-15-keto-prostaglandin F. Biol Reprod 1986; 35:312-9.

Singh SK, Dominic CJ. Prostaglandin $F_{2\alpha}$-induced changes in the sex organs of the male laboratory mouse. Exp Clin Endocrinol 1986; 88(3):309-15.

Siiteri PK. Adipose tissue as a source of hormones. Am J Clin Nutr 1987 [Review].

Smith WL, Borgeat P. The Eicosanoids: Prostablandins, Thromboxanes, Leukotrienes, and Hydroxy-Eicosaenoic Acids. In: Vance DE, Vance JE (eds.), Biochemistry of Lipids and Membranes, Menlo Park, California: The Benjamin/Cummings Publishing Co. Inc.; 1985:325-360.

Soto EA, Kliman HJ, Strauss JF 3d, Paavola LG. Gonadotrophins and cyclic adenosine 3',5'-monophosphate (cAMP) alterthe morphology of cultured human granulosa cells. Biol Reprod 1986; 34(3):559-69.

Spilman CH, Harper MJ. Effects of prostaglandins on oviductal motility and egg transport. Gynecol Invest 1975; 6:186-205 [Review].

Srivastava RK, Luu TV, Marrone BL, Harris HS, Sridaran R. Inhibition of steroidogenesis by luteal cells of early pregnancy in the rat in response to in vitro administration of a gonadotropin-releasing hormone agonist. J Steroid Biochem Mol Biol 1994; 49(1):73-9.

Steele GL, Leung PCK. Mechanism of prostaglandin $F_{2\alpha}$ action in the ovary. J Lipid Mediat 1993; 6:509-13.

Stoljelkovics SS, Reinhart J, Catt KJ. Gonadotropin-releasing hormone receptors: Structure and signal transduction pathways. Endocrine Reviews 1994; 15:462-499.

Stryder L. Biochemistry, New York: WH Freeman and Company; 1988:555-574, 991-1000.

Suginami H, Okamura H, Yogo I. In vitro steroidogenesis by human corpora lutea of pregnancy. Effects of human chorionic gonadotropin and prostaglandin $F_{2\alpha}$. Obstet Gynecol 1976; 47(2):177-82.

Sugino H, Nakamura T, Hasegawa Y, Miyamoto K, Igarashi M, Eto Y, Shibai H, Titani K. Identification of a specific receptor for erythroid differentiation factor on follicular granulosa cell. Journal of Biological Chemistry 1988; 263(30):15249-52.

Suzuki M, Takahashi K. Hypothalamo-hypophyseal control of ovulation. pp. 114-21. In: Hatotani N, ed. Psychoneuroendocrinology 1974; Basel, Karger [Review].

Takahashi H, Duleba AJ, Yuen BH, Moon YS. Steroidogenic capabilities of various compartments of rat ovarian follicles in culture. Steroids 1984; 44:337-48.

Tonetta SA, diZerega GS. Intragonadal regulation of follicular maturation. Endocr Rev 1989; 10:205-29 [Review].

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand PT, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, et al. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase. C J Biol Chem 1991; 266:15771-81.

Tsang BK, Moon YS, Armstrong DT. Estradiol-17 beta and androgen secretion by isolated porcine ovarian follicular cells in vitro. Can J Physiol Pharmacol 1982; 60:1112-8.

Turgeon JL. Neural control of ovulation. Physiologist 1980; 23(3):56-62 [Review].

Tureck RW, Mastroianni Jr L, Blasco L, Strauss JR. Inhibition of human granulosa cells progesterone secretion by gonadotropin releasing hormone agonist. J Clin Endocrinol Metab 1982; 54:1078-1080.

Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971; 231:232-5.

Waite M. Phospholipases. In: Vance DE, Vance JE (eds.), Biochemistry of Lipids and Membranes, Menlo Park, California: The Benjamin/Cummings Publishing Co. Inc.; 1985:299-324.

Wallace CR, Kiser TE, Rampacek GB, Draeling RR. The relationship between 13, 14-dihydro-15-keto $PGF_{2\alpha}$ and LH secretion in bulls. Prostaglandins 1985; 30(6):925-33.

Wang J, Leung PC. Role of arachidonic acid in luteinizing hormone-releasing hormone action: stimulation of progesterone production in rat granulosa cells. Endocrinology 1988; 122(3):906-11.

Watanabe H, Nagai K, Yamaguchi M, Ikenoue T, Mori N. Interleukin-1 beta stimulates prostaglandin E2 and $F_{2\alpha}$ synthesis in human ovarian granulosa cells in culture. Prostaglandins Leukot Essent Fatty Acids 1993; 49:963-7.

Watson J, Shepherd TS, Dodson KS. Prostaglandin E-2-9-ketoreductase in ovarian tissues. J Reprod Fertil 1979; 57:489-96.

Webley GE, Richardson MC, Summers PM, Given A, Hearn JP. Changing responsiveness of luteal cells of the marmoset monkey (Callithrix jacchus) to luteotrophic and luteolytic agents during normal and conception cycles. J Reprod Fertil 1989; 87:301-10.

Webley GE, Richardson MC, Given A, Harper J, Preincubation of human granulosa cells with gonadotrophin prevents the cloprostenol-induced inhibition of progesterone production. Hum Reprod 1991;6(6):779-82.

Whitelaw PF, Eidne KA, Sellar R, Smyth CD, Hillier SG. Gonadotropin-releasing hormone receptor messenger ribonucleic acid expression in rat ovary. Endocrinology 1995; 136(1):172-9.

Wierman ME, Gharib SD, Chin WW. The structure and regulation of the pituitary gonadotrophin subunit genes. Baillieres Clinical Endocrinology & Metabolism 1988; 2(4):869-89 [Review].

Williams WF, Lewis GS, Thatcher WW, Underwood CS. Plasma 13,14-dihydro-15 keto $PGF_{1\alpha}$ (PGFM) in pregnant and nonpregnant heifers prior to and during surgery and following intrauterine injection of $PGF_{2\alpha}$. Prostaglandins 1983; 25(6):891-9.

Wu CH, Prazak LM. Endocrine basis for ovulation induction. Clinical Obstetrics & Gynecology 1974; 17(4):65-78 [Review].

Yen SS. Regulation of the hypothalamic--pituitary--ovarian axis in women. Journal of Reproduction & Fertility 1977; 51(1):181-91.

Ying SY, Becker A, Swanson G, Tan P, Ling N, Esch F, Ueno N, Shimasaki S, Guillemin R. Follistatin specifically inhibits pituitary follicle stimulating hormone release in vitro. Biochemical & Biophysical Research Communications 1987; 149(1):133-9.

Yuh KC, Possley RM, Brabec RK, Keyes PL. Steroidogenic and morphological characteristics of granulosa and thecal compartments of the differentiating rabbit corpus luteum in culture. J Reprod Fertil 1986; 76(1):267-77.

Xiao S, Robertson DM, Findlay JK. Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 1992; 131(3):1009-16.

Zolti M, Meirom R, Shemesh M, Wollach D, Mashiach S, Shore L, Rafael ZB. Granulosa cells as a source and target organ for tumor necrosis factor-alpha. Febs Lett 1990; 261:253-5.