Bo L. Dennefors

Studies on the endocrine function of the human ovary

Experiments conducted on isolated ovarian compartments

Göteborg 1981

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ABSTRACT

Dennefors, B., Studies on the endocrine function of the human ovary. Experiments conducted on isolated ovarian compartments. Department of Obstetrics and Gynecology, University of Göteborg, 413 45 Göteborg, Sweden.

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In the preovulatory follicle both the granulosa and the theca cells had the capacity to produce all three steroids measured in vitro. The dominant steroid formed was P by granulosa cells and A by theca cells. E_2 was formed in considerable amounts by both cell types. These results demonstrate that steroidogenesis is not strictly compartmentalized in the human preovulatory follicle. Both cell types responded to hCG with increased cAMP and P formation. Thus, in the preovulatory follicle the granulosa cells develop a responsiveness to LH/hCG, which is of essential importance for normal follicular function and luteinization.

In pieces of precisely dated human CL, hCG was found to stimulate cAMP and P formation at all ages of the CL. $PGF_{2\alpha}$ counteracted the stimulatory effect of hCG on both cAMP and P formation in CL of the mid-luteal phase, strongly indicating a luteolytic effect of $PGF_{2\alpha}$ in the human.

Specimens from the stromal compartment of postmenopausal ovaries produced measurable amounts of all three steroids measured, with A being the major steroid formed. Hyperplastic stroma produced 2-fold higher amounts of A and E_2 than atrophic stroma. Human CG had no effect on cAMP accumulation in atrophic stroma, while in hyperplastic stroma hCG elicited a marked increase in cAMP formation, indicating a responsiveness to gonadotropin in this type of ovarian stroma.

Hilus cells from postmenopausal ovaries showed a similar pattern of steroidogenesis in vitro to postmenopausal stroma, but the amounts of steroids formed were clearly higher in hilus cells. The hilus cells responded to hCG with increased cAMP and steroid formation. It is suggested that the hilus cells form an additional functional compartment of the human ovary.

Key words: ovary, human, theca cells, granulosa cells, corpus luteum, stroma, hilus cells, progesterone, androstenedione, estradiol, cyclic AMP, gonadotropin, prostaglandins.

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CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Steroid and cyclic AMP formation in granulosa and theca cells from human preovulatory follicles in response to hCG.
Dennefors B, Nilsson L and Hamberger L. J Clin Endocrinol Metab, accepted for publication.

II. Progesterone and cyclic AMP formation by isolated human corpora lutea of different ages: influence of human chorionic gonadotropin and prostaglandins.
Dennefors B, Sjögren A and Hamberger L. J Clin Endocrinol Metab, submitted for publication.

III. Steroid production and responsiveness to gonadotropin in isolated stromal tissue of human postmenopausal ovaries.
Dennefors B, Janson PO, Knutson F and Hamberger L. Am J Obstet Gynecol 136:997, 1980.

IV. Hilus cells from human postmenopausal ovaries: gonadotropin sensitivity, steroid and cyclic AMP production.
Dennefors B, Janson PO, Hamberger L and Knutson F. Acta Obstet Gynecol Scand, accepted for publication.

The papers will be referred to in the text by their Roman numerals.

LIST OF ABBREVIATIONS

A	androstenedione
Ag	aminoglutethimide
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	adenosine-(cyclic)-3', 5'-monophosphate (cyclic AMP)
CL	corpus luteum, corpora lutea
cpm	counts per minute
E_2	estradiol-17β
FSH	follicle-stimulating hormone
g	gravity
hCG	human chorionic gonadotropin (human CG)
hPL	human placental lactogen
IU	international unit
KRB	Kreb's Ringer bicarbonate buffer
LH	luteinizing hormone
LHRH	LH releasing hormone
LMP	last menstrual period
NE	norepinephrine
NS	non significant
P	progesterone
PCO	polycystic ovary syndrome
PG(:s)	prostaglandin(s)
PMSG	pregnant mares´serum gonadotropin
r	correlation coefficient
refs	references
RIA	radioimmunoassay
SEM	standard error of the mean
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)-aminomethane

INTRODUCTION

As a part of the female reproductive system the human ovary has a dual function: the production of germ cells and the secretion of sex steroid hormones. During the fertile period of life the endocrine activity of the ovary undergoes regular cyclic changes, which can be regarded as periodic preparations for fertilization and pregnancy, while around the menopause the endocrine function of the ovary changes dramatically.

The endocrine activity of the ovary is located in different functional compartments with distinct morphological and functional characteristics. These compartments, however, are intimately linked to each other for modulation of the total hormonal secretion from the ovary.

Morphology

The ovary of adult women consists of three reasonably well defined compartments: the follicle, the corpus luteum (CL) and the stroma. Under the thin germinal epithelium covering the surface of the ovary and a more or less well defined layer of dense connective tissue (tunica albuginea), there is a thick layer of special connective tissue (cortex) in which are embedded the various sized follicles, the CL and the corpora albicantes. This interstitial connective tissue of the cortex (cortical stroma) is composed of networks of reticular fibres and spindle shaped cells, resembling smooth muscle cells (Bloom and Fawcett 1962). In addition, non-vascular autonomic nerves have been demonstrated in the stroma (Owman et al 1967).

The mature follicle is a large vesicle containing the yellowish follicular fluid surrounded by a capsule of two layers: the theca interna, highly vascularized with loosely arranged, large cells and the theca externa, with a dense structure of concentrically arranged, fusiform cells. The theca externa layer also contains smooth muscle cells in close contact with adrenergic and cholinergic nerve terminals (Owman et al 1975). There is no sharp limit between the theca externa and the surrounding cortical stroma, while the theca interna is separated by a basement membrane from the follicular epithelium lining the cavity (membrana granulosa). This follicular epithelium consists of several layers of polyhedral cells (the granulosa cells) with no vascularization. The membrana granulosa is thickened on one side of the follicle (cumulus oophorus) where it surrounds the ovum.

After rupture of the follicle and the discharge of the follicular fluid and the ovum with its cumulus oophorus, the follicle is transformed into a

CL. The granulosa and theca interna cells hypertrophy markedly, the nuclei enlarge and the cells gain lipoid material (luteinization). Accompanying CL development there is an intense vascular development throughout the granulosa layers which, like the theca cells, become surrounded by a network of sinusoidal blood capillaries. The free space in the center of the CL is filled with the remains of the follicular fluid, transuded serum and erytrocytes (Bloom and Fawcett 1962).

The ovarian cortex surrounds a medulla consisting of loose connective tissue (medullary stroma), large blood vessels and strands of smooth muscle fibres which extend into the ovary from its medial edge, the hilus. In the hilus of the ovary groups of large, epitheloid cells can be found in close connection with non-myelinated nerve fibres. These cells, which are morphologically identical to testicular Leydig cells, are generally referred to as hilus or hilar cells (Sternberg 1949).

In the years around the menopause, the remaining follicles and CL gradually degenerate and disappear, while the stroma often shows nodular or diffuse proliferation (cortical stromal hyperplasia; see Fig 2, paper III). After menopause the hilus cells also may become more prominent (Boss et al 1965).

Endocrine function

The main endocrine function of the human ovary is to produce and secrete steroid hormones: progestins, androgens and estrogens. This ovarian function has been studied indirectly by measuring sex steroid hormones in urine and peripheral blood during various stages of the menstrual cycle, after the menopause and after bilateral oophorectomy (for refs see Gurpide 1976). Since the adrenals also contribute substantially to the production of sex steroid hormones in the female, hormone determinations after supression of adrenal function or following adrenalectomy have given more selective information on the endocrine function of the ovary (for refs see Gurpide 1976). Human experiments, using infusion of exogenous gonadotropins followed by serial analyses of hormone levels in peripheral blood, have provided further knowledge of the endocrine capacity of the human ovary (e.g. Greenblatt et al 1976). In the last decades, analyses of hormones in the ovarian vein blood (e.g. Aedo et al 1980) have given more direct information on the hormonal secretion of the ovary. However, in vivo studies of this type only reflect the total sum of the hormones secreted by the different ovarian compartments. In order to study the function of the individual compartments, in vitro studies of well defined, isolated specimens from the ovary are

necessary. The vast majority of such studies have been performed in rats and other non-primate species, and have given new information and insight into the functional complexity of the different ovarian compartments.

Our current knowledge of the endocrine function of the ovary may briefly be summarized as follows.

During the follicular phase the human ovary secretes estrogens (mainly estradiol-17β and estrone) in slowly increasing amounts followed by a dramatic acceleration the last four days of the follicular phase. Progesterone (P) is released in low amounts throughout this phase. The major ovarian source of these steroid hormones is the growing follicles, especially the large, preovulatory follicle. The site of steroidogenesis within the follicle is not fully clear to date. In several animal species (e.g. the rat) there is evidence that steroidogenesis is compartmentalized between the granulosa and theca cells, and that the theca cells produce and secrete mainly androgens which, in turn, are transported through the basement membrane and aromatized to estrogens by the granulosa cells. This so called "two cell" hypothesis of follicular estrogen secretion originating from Falck's work in the rat (Falck 1959) has been a matter of controversy, however, during the last decades and specific species differences seem to exist (for refs see Richards 1980).

Starting on the day before ovulation, P secretion rapidly rises during the luteal phase, and reaches its peak value around day 8 after ovulation. During the last 3-4 days of the luteal phase, P secretion quickly drops again, reflecting the normal regression of the CL. Consequently, almost all P secreted by the ovary during the luteal phase is derived from the CL. At the time of ovulation, the secretion of estrogens quickly declines to levels similar to those of the mid-follicular phase, whereafter a new, gradual increase in estrogen secretion takes place. This second peak of estrogen output reaches its maximum around the mid-luteal phase and then gradually declines. Estrogens secreted by the human ovary during the luteal phase are produced predominantly by the active CL, but to some extent also by the follicles.

Also the secretion of androstenedione (A), the major androgen produced by the human ovary, fluctuates throughout the cycle with the highest amounts in the late follicular and early luteal phases. It appears that all three functional compartments of the ovary contribute to the total ovarian secretion of A (Baird et al 1974).

After menopause, the normal ovary secretes only minimal quantities of estrogens and P as judged by hormone levels in peripheral and ovarian venous

plasma (Judd et al 1974, Vermeulen 1976). However, the amounts of A and testosterone in the ovarian vein blood from postmenopausal women are almost within the same range as in premenopausal women (Greenblatt et al 1976). These findings indicate that the postmenopausal ovary secretes mainly androgens. Several in vitro studies have revealed that steroidogenesis in the postmenopausal ovary takes place in the stromal compartment (Rice and Savard 1966, Mattingly and Huang 1969), and possibly also in the hilus cells (Scully and Cohen 1964).

Gonadotropic regulation

The endocrine function of the ovary is regulated by gonadotropic hormones, which are essential for normal growth and development of the ovary. The gonadotropins in the cyclical woman are FSH, LH and prolactin and, during pregnancy, hCG and hPL also can be included. According to the classical view, FSH stimulates growth and ripening of the follicle and, in combination with LH, elicits full follicular maturation. The mid-cycle LH surge initiates follicular rupture and CL formation. The function and survival of the CL thereafter is maintained by LH or hCG.

In the last decade, results from numerous studies, both in vivo and in vitro, have modified this simple description of the gonadotropic actions, and the regulation of steroidogenesis in the ovary appears to be a complex system of steroid and protein hormone actions and interactions on the target cells. The present working hypothesis for the regulation of ovarian steroidogenesis can briefly be summarized as follows.

In the developing follicle FSH stimulates estradiol-17β (E_2) synthesis in the granulosa cells by activating the aromatase enzyme system (Moon et al 1978), and, as a consequence, increasing levels of not only FSH but also E_2 are accumulating in the antral fluid. LH, on the other hand, stimulates the theca cells to an increased synthesis of androgens (Tsang et al 1979), which provide substrate for estrogen production. During the late follicular phase, when LH in serum rises to peak concentrations, this gonadotropin induces ovulation and stimulates luteinization of both theca and granulosa cells for the consequent formation of a CL. The role of prolactin in regulating the steroidogenesis in the follicle is unclear, but it has been shown that high levels of prolactin in blood (hyperprolactinemia) and in follicular fluid are associated with follicular atresia and depressed steroid secretion (McNatty et al 1974, McNatty 1979).

As pointed out above, the CL needs support of LH for its survival (Vande Wiele 1970) and LH/hCG stimulates steroidogenesis in the CL. In

several non-primate species prolactin also is necessary for the maintenance and secretory activity of the CL (for refs see Rothchild 1981), while the role of prolactin for human luteal function remains somewhat controversial. If fertilization does not occur, the steroid secretion of the CL begins to decline by day 10-12 after ovulation, despite the fact that the CL is still exposed to certain amounts of circulating LH. The reasons for this so called functional luteolysis are still unclear. However, there is now strong evidence in several animal species that local actions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the ovary induce luteolysis (see below).

Little is known about the role of gonadotropins for steroid production in the stromal compartment, but there are some indications that at least LH/hCG stimulates steroidogenesis in the ovarian stroma of both fertile (Rice and Savard 1966) and postmenopausal human ovaries (Poliak et al 1968).

The mechanisms by which the gonadotropins act to regulate steroidogenesis in their target cells in the ovary are still not known in detail. However, the initial step in the action of these protein hormones appears to involve binding of the hormone to a specific receptor on the cell membrane. Binding to ovarian tissues is hormone-specific with the exception that LH and hCG probably compete for the same receptor.

In the ovarian follicle of the rat, the theca cells have only LH/hCG receptors, while FSH receptors are located exclusively on the granulosa cells (Richards et al 1976). This latter cell type in the human has also LH/hCG receptors, the concentration of which increases during follicular development (Rajaniemi et al 1981). Furthermore, receptors for prolactin also have been detected on the granulosa cells of ovarian follicles in the pig (Rolland and Hammond 1975). The human CL has been found to contain receptors for LH/hCG (e.g. Lee et al 1973) as well as for prolactin (McNeilly et al 1980).

After binding of the gonadotropin to its receptor, the membrane bound enzyme adenylate cyclase (AC) is activated which in turn catalyzes conversion of ATP to cyclic 3', 5'-adenosine monophosphate (cAMP).

Role of the cAMP system

Both FSH and LH/hCG have been shown to increase intracellular levels of cAMP in various ovarian cell types, and furthermore, an increase in cAMP formation after exposing ovarian specimens to gonadotropins is generally accepted as an indication of responsiveness to the gonadotropin. This increase in intracellular cAMP after stimulation with gonadotropins in vitro is a rapid process occurring within minutes (Nilsson et al 1974).

There is, to date, overwhelming evidence to support the "second messenger" theory which implicates cAMP as an intracellular mediator for gonadotropic effects (Rall and Sutherland 1958). Within the cell, cAMP exerts its effect on steroidogenesis in a complex and incompletely understood way. However, it is known that cAMP binds to and activates a protein kinase which causes phosphorylation of protein substrates that regulate the functional responses in the target cell (Davies et al 1978).

Other regulatory factors

All dynamic changes in the metabolism and steroid production of the various compartments of the ovary during the reproductive cycle are not exclusively regulated by gonadotropins or mediated by cAMP. Several local feed-back systems for steroidogenesis have been suggested. Such systems include local control by ovarian steroid hormones of certain steps in the steroidogenesis of the ovary plus a variety of intrafollicular peptide regulators such as inhibin (for refs see Channing 1979), FSH-binding inhibitor (Darga and Reichert 1978) and luteinizing inhibitor (Ledwitz-Rigby et al 1977). Furthermore, locally formed peptides with LHRH-like activity (gonadocrinins) have been suggested to influence ovarian function (Hsueh and Erickson 1979, Ying and Guillemin 1980), and the autonomic innervation of the ovary is probably also implicated in these regulatory systems (e.g. Owman et al 1967, Walles 1977, Burden 1978). The significance of these suggested local regulatory mechanisms remains, however, a matter of debate and further studies have to be performed to clarify their physiological importance.

The role of prostaglandins (PG:s) in regulating steroidogenesis in the ovary is another exiting field of exploration in the context of regulatory factors. PG:s of the E-type were originally proposed as obligatory mediators of LH induced stimulation of cAMP accumulation and steroidogenesis (Kuehl et al 1970), but later studies have contradicted this hypothesis (for refs see Zor and Lamprecht 1977). Nowadays, the PG:s of the E-type are regarded more as modulators of gonadotropic effects. Another compound belonging to the group of classical PG:s, $PGF_{2\alpha}$, has been shown to induce luteolysis when administered to pseudopregnant rats, guinea pigs, hamsters, rabbits and rhesus monkeys (for refs see Horton and Poyser 1976). Attempts to demonstrate a luteolytic effect of $PGF_{2\alpha}$ by systemic administration to humans have so far been fruitless, at least when given in tolerable doses (e.g. Jewelewicz et al 1972, LeMaire and Shapiro 1972). Therefore, at present, it is not known if $PGF_{2\alpha}$ is luteolytic also in the human.

AIM OF THE PRESENT STUDY

The research laboratory of this department (Dept. of Obstetrics and Gynecology) is working in close connection with the Endocrine division, Dept. of Physiology, University of Gothenburg. In this latter laboratory, extensive studies on the metabolism of the ovary and its gonadotropic control have been performed during the last 20 years, mainly using the isolated rat ovary and its different compartments as experimental models. However, since there is a great variability in the ovarian endocrine function and its regulation between different species, results from experiments on rats and other laboratory animals cannot uncritically be extrapolated to the human situation. On the other hand, most of our current knowledge of human ovarian function originates from experiments on laboratory animals, from which the results have been confirmed or modified in subsequent human experiments. In the last five years, our main purpose has been to transfer the detailed knowledge of experimental designs and results from studies on the rat ovary to experiments on the human ovary. The experimental techniques and analyses used in the present study are thus similar to those which have been applied in previous animal models.

Most earlier studies on the in vitro metabolism of isolated compartments from the human ovary have, with few exceptions, utilized cells or tissues from poorly characterized patients. However, exact estimations of the phase of the cycle are of crucial importance for interpretation of the results, since ovarian steroidogenesis is a dynamic system, undergoing continuous changes throughout the life span of the follicle or CL. Furthermore, when studying the endocrine activity of a specific compartment of the human ovary, it is necessary to characterize the isolated specimens morphologically to ensure that there is no contamination from other cell types and to identify possible signs of cellular abnormalities (degeneration, hyperplasia, neoplasia). Consequently, two basic aims of the present work were to isolate and morphologically characterize the various cellular compartments of the human ovary, and to analyse their in vitro steroid formation. The tissues were obtained from women who were carefully classified as regards the phase of the menstrual cycle or the postmenopausal period.

Most earlier studies on the steroidogenic capacity of the human ovary have used long term tissue culture as experimental model. However, it is well known that the different cell types of the ovary may change morphologically and probably also functionally during culture. Furthermore, in long

term incubations, the availability of substrate and cofactors needed for steroidogenesis can never be exactly the same as in the in vivo situation. Therefore, in the present study we have used short term incubation periods (1-4 h), which are probably more "physiological" in this respect.

The influences of gonadotropins on the human ovary vary markedly between different ovarian cell types and are dependent on the phase of the menstrual cycle. Therefore, another aim of the present study was to test the responsiveness of the various ovarian cell types to a gonadotropin (hCG), by measuring the acute effects on steroidogenesis and cAMP formation. In addition, comparisons were made between these effects in the different compartments of the human ovary. Human CG was chosen since it is a commercially available pure gonadotropin with biological effects very similar to LH.

The mechanism underlying the normal regression of the CL of the cycle has been an attractive field of research during the last decade, probably because of its importance in relation to the control of fertility. In several animal species $PGF_{2\alpha}$ appears to be of physiological importance for induction of luteolysis (for review see Horton and Poyser 1976). In the human, attempts to demonstrate a luteolytic effect of $PGF_{2\alpha}$, however, have yielded inconsistent results. Another aim of the present study was to determine whether PG:s (especially $PGF_{2\alpha}$) are luteolytic also in the human. This possible luteolytic action was investigated by analyzing the influence of PG:s, in combination with hCG, on cAMP and P formation in isolated human CL of different ages.

The hilus cells of the human ovary show histological and histochemical signs of secretory activity, and further, these cells are particularly prominent during pregnancy and around the menopause, possibly indicating responsiveness to gonadotropins. Based on these findings it has been suggested that the ovarian hilus cells produce steroid hormones (Sternberg 1949). However, since no direct in vitro study of the proposed endocrine activity of these cells had been performed, the role of hilus cells in the steroid production of the human ovary was of specific interest to explore. The final aim of the present work was therefore to test microscopically defined specimens from the hilus area of human postmenopausal ovaries for their steroidogenic capacity and gonadotropic responsiveness in vitro.

In summary the purpose of the present study has been:

1) to isolate and characterize cells from various compartments of the human ovary.
2) to study specific aspects of steroidogenesis in granulosa and theca cells from preovulatory follicles, in CL of different ages, and in stromal and hilus cells from postmenopausal ovaries.
3) to study gonadotropic influence on cAMP and steroid production in these various cell types derived from fertile and postmenopausal ovaries.
4) to study the regulatory effects of PG:s on the function of the CL with special reference to luteolysis.

METHODOLOGICAL CONSIDERATIONS

Patients

Ovarian tissues were obtained from women undergoing laparotomy for various gynecological reasons. In paper I and II legal sterilization by tubal ligation was the reason for surgery. All patients in these two studies had regular menstrual cycles and had no hormonal therapy for a minimum of 6 weeks prior to the operation. Estimation of the phase of the cycle was based on the following parameters: 1/ serum levels of P, E_2 and LH taken 1-2 days before, during, and 2 and 5 days after the operation,
2/ histological examination of an endometrial biopsy taken at operation,
3/ day of last menstrual period (LMP).
By combining these data, the day of the menstrual cycle at the time of surgery could be determined with a margin of $\pm$ 24 h. Data from a typical patient are illustrated in Fig. 1.

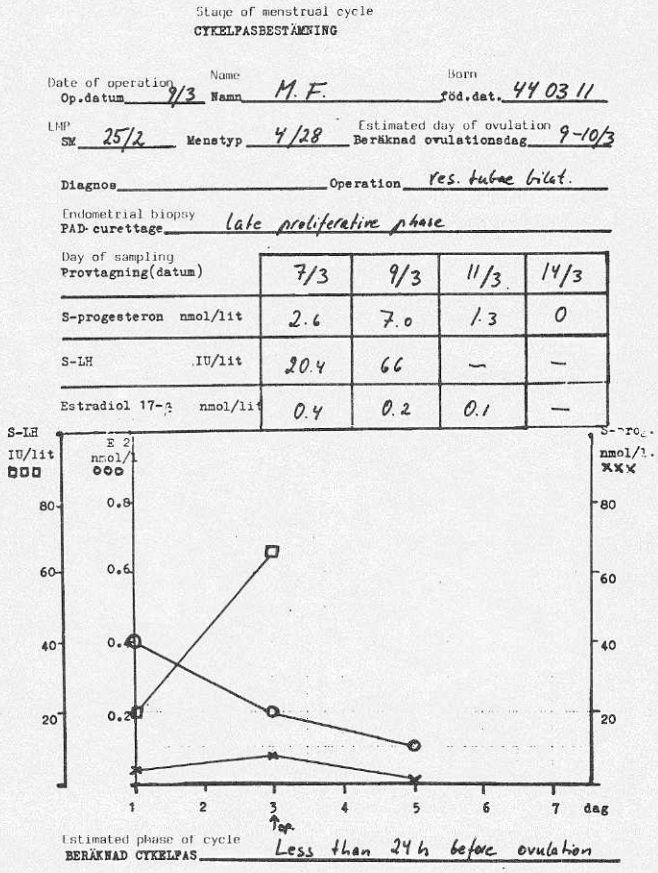

Fig. 1. Estimations of menstrual cycle phase. Summary of data from a typical patient including hormone blood levels, histology of endometrial biopsy and day of LMP. This patient was operated on day 3 and her hormone levels were indicative of ovulation within 24 h of the operation.

All patients in paper III and IV were more than two years after the menopause; the lack of follicular function was confirmed by elevated serum levels of FSH (> 50 IU/l). These patients were operated on for various gynecological disorders (fibroids, benign unilateral ovarian tumor, hydrosalpinx, atypical cells from cervix or corpus uteri and, in one case, cancer of the uterine body). None of the patients had received radiotherapy or hormonal treatment within six months before surgery. Both ovaries were routinely removed and all macroscopically normal ovaries were used for the incubations.

Isolation and incubation procedures

Preovulatory follicles (paper I): A small wedge resection of one ovary containing the largest and most protruding follicle was performed during the operation, and the ovarian biopsy was immediately transported to the laboratory in Krebs Ringer bicarbonate buffer (KRB) (see below) at room temperature. The follicle was then dissected free from surrounding tissues, the diameter was measured and the granulosa and theca cells were isolated as described in detail in paper I. No attempt was made to separate the inner layers from the outer layers of granulosa cells, although there is evidence for a functional gradient within the membrana granulosa (Richards 1980). In all experiments the granulosa cells were counted in a haemocytometer and their viability was tested with 0.4% Trypan blue vital stain. In some experiments histologic examination of the residual theca capsule was performed in order to check its purity (see below). The isolated cells were preincubated for 5 min and incubated for 30-240 min.

Corpus luteum (paper II): The corpus luteum was excised in toto, placed in ice-chilled KRB and transported to the laboratory. The corpus luteum was then separated from adjacent tissues and dissected radially into small pieces, each with an approximate wet weight of 5-20 mg. These specimens were then preincubated for 60 min and incubated for 5-120 min. In certain experiments pieces of the corpus luteum were taken before incubation for histologic examinations (see below).

Cortical stroma (paper III): Immediately after removal the postmenopausal ovaries were placed in ice-cold KRB and transported to the laboratory. The outer layer (2 mm) of the ovarian cortex was dissected free and the remaining cortical tissue was cut into small pieces, each approx. 1-2 mm in size and a wet weight of 2-4 mg. Each piece of tissue was then cut into two

halves, one of which was fixed for histologic examination (see below). The other half was preincubated for 30 min and incubated for 240 min.

Hilus cells (paper IV): The postmenopausal ovaries were collected in the operating room and transported to the laboratory. Strips of hilus tissue (5x25 mm in size) adjacent to the ovarian margin were dissected free and divided into two halves, one of which was used for histologic characterization (see below). The other half was cut into numerous small pieces (approx. 1x1 mm in size) which were preincubated for 30 min and then incubated for 30 or 240 min.

Incubation medium: All incubations were performed in Krebs Ringer bicarbonate buffer (see Umbreit et al 1964), pH 7.4, containing 1 mg/ml (5.5 mM) glucose and 0.1% (paper I) or 1% (paper II, III and IV) fatty acid free bovine serum albumin. A modification of the buffer was made by reducing the calcium concentration from 2.5 mM to 1.25 mM in order to obtain a more physiological concentration of free calcium ions. The buffer was oxygenated (95% O_2 + 5% CO_2) at the start of the preincubation period and at the start of the incubation period to maintain optimal buffer capacity. When incubation time was 4 h, the incubation flasks were regassed after 2 h of incubation.

General characteristics of isolated ovarian tissues

Attempts were made to identify and morphologically characterize the different ovarian compartments which were isolated and incubated. The tissues were fixed in neutral 4% formaldehyde solution and light microscopical examinations were performed.

Preovulatory follicles: The follicular size and the status of the oocyte are described in paper I. The number of granulosa cells varied with the diameter of the follicles: minimum diameter 12 mm - 7 million cells, maximum diameter 30 mm - 40 million cells. The viability of the granulosa cells, as estimated with Trypan blue stain, was found to be 60 - 70%. Each aliquot of incubated cells contained approx. 0.5 million viable granulosa cells.
The residual theca capsule was carefully checked for contamination of granulosa cells. These theca capsules were found generally to be free from contaminating granulosa cells with the exception of occasional small patches made up of 1-2 layers of granulosa cells.

Corpus luteum: The corpus luteum could easily be identified macroscopically. In certain experiments portions of the corpus luteum were examined histologically and were found to be normal. Attempts to classify the age of the corpus luteum based on histologic appearance of the luteinized cells were discarded, since this method proved to give less exact estimation of the age of the corpus luteum compared to the other utilized methods.

Cortical stroma: Since the ovarian stroma is, macroscopically, a less distinguishable compartment than the follicle or the corpus luteum, the opposite half of each incubated piece of stroma tissue was examined histologically to ensure that no other active ovarian structures were included. According to the structure of the stroma the cases could be divided into two groups: "normal", atrophic stroma and stromal hyperplasia. For further details see paper III.

Hilus cells: The hilus cells are difficult to identify in small specimens from the hilus region, since these cells are located in irregularly scattered nests throughout the hilus. For such reasons the intact half of the whole strip from the hilus was examined histologically. Only cases where this half of tissue showed clusters of typical hilus cells and no fragments of cortical or medullary stroma were included in the study. Furthermore, since the other structures of the hilus (i.e. muscle cells, vessels, nerves, connective tissue) are not known to produce any of the steroids measured, and since all specimens selected were found to release measureable amounts of steroids into the incubation medium, this technique to identify hilus cells seems feasable.

Hormone preparations

A general problem in experiments dealing with hypophyseal gonadotropic hormones is the difficulty in obtaining pure preparations. One aim of the present study was to test the effects of one gonadotropin on the various compartments of the ovary. Human CG was chosen since it was possible to obtain it in a very pure form. Human CG competes for the same receptors as LH and has many similar effects. It must be kept in mind, however, that there are also differences in the biological, especially long-term, effects of hCG and LH both in experimental investigations and in clinical practice (e.g. Ahrén et al 1980). Highly purified human CG was a gift from Leo Ltd., Helsingborg, Sweden (activity 6000 IU/mg in paper III, 10000 IU/mg in paper I, II and IV). Prostaglandin E_2 and $F_{2\alpha}$ (paper II) dry substance (a gift from

Upjohn Co., Kalamazoo, USA) were dissolved in 95% ethanol (stock solution 1 mg/ml). Immediately prior to use, these PG solutions were diluted with buffer to give a final concentration of 1 µg/ml (ethanol conc. 0.1%). In certain experiments on human CL only the ethanol vehicle was added to the incubation medium in the final concentration of 0.1%. It was found that this amount of ethanol did not interfere with the steroid or cAMP formation in vitro.

Determination of cAMP

Following incubation the ovarian specimens were homogenized in 1.2 ml 5% trichloroacetic acid (TCA). Samples of the homogenates were taken for determination of protein content according to Lowry et al (1951). After centrifugation 50 µl of HCl was added to the supernatant and the TCA was removed by washing the samples with dietyl ether 6 times. The aqueous phase was evaporated to dryness under a stream of air, and the residue was dissolved in 0.05 M sodium acetate buffer and used directly for the cAMP assay. Cyclic AMP was determined according to the protein binding assay of Gilman (1970) with the following modifications: albumin 1% was used instead of protein kinase inhibitor, and dextran coated charcoal instead of millipore filter for the separation of bound and free cAMP. Standards ranging from 0.5 - 100 pmol were assayed together with the unknown samples in duplicates. The unknown samples were calculated from the standard curve which was expressed using the logit-log transformation.

Steroid formation in vitro

The steroids progesterone (P), androstenedione (A) and 17β-estradiol (E_2) were measured since these three steroids represent the three major groups of sex-steroid hormones produced by the human ovary. The release of these steroids into the incubation medium during various incubation periods and the content in follicular fluid were measured by radioimmunological technique (RIA).

In certain experiments (on theca and luteal cells) the release of estrone into the incubation medium also was measured by RIA. A good correlation between the amounts of estrone and E_2 was then found both for controls and hCG-treated specimens (r= 0.93). However, estrone levels in the incubation media were generally 5 - 10 fold lower than those of E_2.

In one series of experiments the tissue levels of the steroids in granulosa and theca cells from preovulatory follicles were measured and compared to the levels in the incubation medium after the incubation period

(Table I). This comparison showed that the steroid levels in the incubation medium were 2 - 50 fold higher than in the tissue (highest difference for granulosa P and lowest for granulosa E_2).

Table I. Comparisons between steroid amounts in the incubation medium and in the tissue of granulosa and theca cells.

		Treatment	Steroid concentration (ng/mg protein)	
			tissue	medium
GRANULOSA CELLS	progesterone	C	7.2 ± 3.9	221.4 ± 19.5
		hCG (100 IU/ml)	7.3 ± 0.9	409.3 ± 37.4
	androstene-dione	C	0.9 ± 0.2	5.3 ± 0.8
		hCG (100 IU/ml)	2.5 ± 0.4	6.6 ± 0.5
	estradiol	C	25.3 ± 7.1	53.8 ± 10.8
		hCG (100 IU/ml)	34.5 ± 1.4	106.4 ± 14.8
THECA CELLS	androstene-dione	C	7.0 ± 1.7	87.7 ± 18.8
		hCG (100 IU/ml)	5.6 ± 2.0	171.2 ± 23.3

The granulosa cells were derived from a healthy preovulatory follicle (incubation time 4 h) and the theca cells from an atretic follicle (incubation time 2 h). Values are given as mean ± SEM. The amounts of steroids are 2-50 fold higher in medium than in tissue.

To test whether the release of steroids into the incubation medium reflects de novo synthesis or release of preformed steroids, aminoglutethimide, a potent inhibitor of steroidogenesis, was added to the incubation medium of granulosa and theca cells. Aminoglutethimide (Ag Elipten, kindly donated by Ciba-Geigy Pharmaceutical Co, Sweden) was used in a concentration of 1 millimoles/l. The results are shown in Table II. The average inhibition of steroid release was found to be 50% for granulosa cells and 40% for theca cells.

Table II. Effects of aminoglutethimide (Ag) 1 mM on the release of steroids into the incubation medium during a 2 h incubation period of granulosa and theca cells.

		Steroid release (ng/mg protein)				
		C	Ag	hCG (100 IU/ml)	hCG (100 IU/ml) + Ag	average inhib.
GRANULOSA CELLS	progesterone	4.8 ± 0.5	0.8 ± 0.1	-	-	17%
	androstenedione	1.7 ± 0.2	1.2 ± 0.3	-	-	75%
	estradiol	0.2 ± 0.0	0.1 ± 0.1	-	-	50%
THECA CELLS	progesterone	207.5 ± 57.2	74.5 ± 9.3	540.2 ± 265.5	78.6 ± 9.5	25%
	androstenedione	87.7 ± 18.8	49.1 ± 35.8	-	-	57%
	estradiol	18.2 ± 2.6	8.7 ± 2.0	50.1 ± 10.2	16.3 ± 8.3	41%

Values are given as mean ± SEM. The cells were derived from a large human, atretic follicle (not included in the study) of a patient who had been primed with Clomiphene and hCG for oocyte collection.

In certain experiments the incubation media were frozen immediately after the preincubation period and were later analysed for steroids. It was then found that the steroid concentrations in these preincubation media were approx. 50% of the concentrations measured after the 2 h incubation period.

Considering the scope of the methods applied in this study, it is concluded that steroid release from tissues into their incubation media during 2-4 h incubations can be closely correlated with de novo steroid synthesis in the incubated specimens.

Radioimmunoassays of steroid hormones

The three steroids were determined by RIA using antisera which were raised and characterized in the Department of Hormone Research, The Weizmann Institute of Science, Israel. The antisera were prepared by immunizing rabbits with BSA-conjugates of progesterone-11α-hemisuccinate, 17β-estradiol-6-carboxymethyloxime and androstenedione-7-carboxy-ethylthioether. It has previously been demonstrated that these antisera are of high specificity and that the cross-reactivity between important steroid hormones is low (Lindner and Bauminger 1974).

Progesterone: For estimation of progesterone, 25 or 50 µl aliquots of incubation medium were transferred to extraction tubes and were brought to a volume of

500 µl with a solution containing 50 mM Tris-HCL hydroxymethylaminomethane, 100 mM NaCl and 15 mM NaN_3 ("Tris I"), all adjusted to pH 10 with one drop of 1M NaOH and extracted with 10 ml of petroleum ether. The extraction tubes were shaken vigorously for 1 min. The recovery of radioactivity when pure ^3H -progesterone was added to samples before extraction and portions of the extract directly counted was approx. 97%. 500 µl of the ether extracts were transferred to glass tubes and evaporated to dryness under a stream of nitrogen. The dried extract was re-suspended in 400 µl Tris HCl buffer (pH 8) containing 0.3% normal rabbit serum ("Tris II"). The tubes were incubated for 10 min at 60°C and placed on ice. To each sample was then added 200 µl of an antibody-^3H -progesterone mixture prepared immediately before the addition. The amount of ^3H -progesterone (1, 2, 6, 7-^3H -progesterone purchased from the Radiochemical Centre, Amersham, England) was adjusted to give a final concentration of 4000 cpm/600 µl. The radioactive progesterone was regularly re-chromatographed on Sephadex LH-20 column (see below). A stock solution was made in toluene:methanol 85:15 to a concentration of approx. 10^7 cpm/ml and stored at 4°C. When making solutions for an assay, 50 µl of this stock solution was evaporated under nitrogen and the dried residue dissolved in an appropriate volume of Tris II.

After adding the antibody-^3H -progesterone mixture the samples were incubated over night at 4°C. The separation of bound and unbound steroid was achieved by adding 100 µl ice-chilled dextran coated charcoal, mixed, incubated for 20 min at 4°C, and centrifuged at 4000 x g in a swing-out-centrifuge for 30 min at 4°C. After centrifugation the supernatant was rapidly transferred to a scintillation vial and mixed with 3 ml RIA-luma (Lumac B.V.). All samples were counted in a Packard Tric-Carb liquid scintillation spectrometer (model 2450).

Standards were prepared by dissolving unlabelled progesterone (from a stock ethanol solution) in Tris II. The final amount of unlabelled progesterone in the reaction mixture was 0, 6.25 (only in paper I and II), 12.5, 25, 50, 100, 200, 400 and 800 pg in 600 µl. Also blank tubes and tracer tubes were included in the assay to give unspecific binding and total radioactivity respectively. The ratio between total amount of ^3H -progesterone bound (corrected for unspecific binding) and total amount added, was 51.3 $\pm$ 3.8% (mean $\pm$ SEM of 9 separate analyses). The standards and unknown samples were run in duplicates. The unknowns were calculated from the standard curve which was expressed using logit-log transformation.

For measurement of the tissue levels of progesterone in granulosa and theca cells, the incubated specimens were homogenized in 1.0 ml ethanol (99%)

and centrifuged at 1000 x g for 20 min at 4°C. Aliquots of the supernatant were extracted with petroleum ether and the extracts were assayed as described above for the medium.

The specificity of the RIA under the described conditions was analysed by comparing values from crude extracts of incubation medium to values obtained from extracts purified by Sephadex LH-20-chromatography according to Carr et al (1971). The solvent system Heptane 85: Benzene 10: Methanol 5, was used in 8 x 100 mm glass columns containing 0.8 g Sephadex. To correct for procedure losses, pure ^{3}H -progesterone was added before chromatography and a portion of the appropriate fraction was taken directly for liquid scintillation counting. The recovery of radioactive progesterone averaged 89%. As shown in Table III there was good agreement between RIA-values obtained from the crude petroleum ether extract and from the purified fraction (r = 0.94).

Table III. Comparisons of steroid determinations obtained by RIA applied to extracts of incubation media with or without chromatographic purification.

Steroid	without chromatography (ng/mg protein)	with chromatography (ng/mg protein)
Progesterone	108.7	126.4
	152.5	160.2
	200.9	181.3
	181.6	190.1
		r = 0.94
Androstenedione	3.97	4.96
	70.73	53.38
	51.86	61.01
	85.13	111.55
	30.09	37.76
	50.13	45.01
	32.60	36.67
		r = 0.91
Estradiol -17β	2.77	1.82
	4.45	5.05
	12.05	7.08
	9.76	6.53
	9.42	5.55
	11.04	12.07
	13.60	10.13
	48.37	45.70
		r = 0.99

The individual values are derived from several different experiments.

The sensitivity of the assay was determined by assaying aliquots of medium which had been incubated without tissue. The medium blank value was 3.4 ± 0.9 pg (mean ± SEM of 5 separate analyses). The lowest detectable amounts of progesterone in 600 µl was therefore considered equal to the lowest amount of unlabelled progesterone in the standard curve, i.e. 6.25 pg/600 µl (12.5 pg/600 µl in paper III and IV).

The reliability of the method was controlled by including samples from a pool of progesterone (dissolved in ethanol and diluted with Tris I and kept at -20°C). The between-assay coefficient of variation (CV) when analysing aliquots of this pool containing 800 pg progesterone was 16.3% and the average within-assay CV was 8.5% (6 analyses).

In several analyses, different amounts of unlabelled progesterone were dissolved in the albumin-containing incubation medium and processed as described above. The recovery varied between 96 and 100%. One such analysis is shown in Table IV. Based on these findings, recovery experiments were not run routinely for all assays.

By taking different volumes of incubation medium for RIA it was also shown that parallelism existed.

Table IV. Relationship between the amount of steroid added to the incubation medium and that measured by RIA.

ng steroid added	ng steroid measured		
	progesterone	androstenedione	estradiol -17β
0.16	0.20	0.18	-
0.32	-	0.28	0.31
0.63	0.49	-	0.61
1.25	1.77	1.90	1.29
2.50	2.45	3.50	2.60
5	5.15	5.74	4.89
10	9.76	9.21	8.09
20	18.72	21.06	-

Different amounts of unlabelled steroids were dissolved in the albumin-containing medium and incubated for 2 h at 37°C. Aliquots of the medium were extracted for RIA.

Estradiol and androstenedione: The assay procedure for determination of E_2 and A was similar to that described for P with the following exceptions: the extraction of steroids from the incubation medium or homogenate of follicular cells was performed with 5 ml diethylether. After separation the aqueous layer was frozen in an ethanol dry-ice mixture and the ether phase was decanted into conical tubes and evaporated under nitrogen. The dried steroids were re-dissolved in 1 ml acetone and 200 µl of this acetone-extract was transferred to glass tubes and evaporated under nitrogen. Thereafter the steroid was dissolved in 400 µl Tris II and the rest of the assay was proceeded as described for P.

Stock solutions of labelled steroids (2, 4, 6, 7 -^{3}H -E_2 and 1, 2 -^{3}H -A from New England Nuclear, Boston, USA) in toluene:methanol (85:15) were kept refrigerated at 4°C. ^{3}H -E_2 was added to a concentration of 10,000 cpm (approx. 80 pg) / 600 µl and ^{3}H -A to 4000 cpm (60 pg) / 600 µl. The ratio between total amount of bound radioactivity and total amount added was 39.6 ± 2.5% (mean ± SEM, 12 separate analyses) for A and 47.0 ± 2.1% (mean ± SEM, 13 separate analyses) for E_2. The purity of labelled hormones was confirmed by Sephadex LH-20 column chromatography.

The specificity of the two RIA:s was confirmed by comparing values obtained by analysing crude ether extracts and purified fractions of the steroids. Both steroids were purified by Sephadex LH-20 column chromatography as described for P using the solvent system Heptan 85: Benzene 10: Methanol 5 (A) and Benzene 90: Methanol 10 (E_2). As shown in Table III, similar values were found whether crude or purified extracts were analysed for both steroids.

The sensitivity of the RIA:s was 11.2 ± 3.0 pg/600 µl (mean ± SEM, 3 separate analyses) for E_2 and 10.6 ± 2.1 pg/600 µl (mean ± SEM, 5 separate analyses) for A.

The reliability of both assays was analysed as described for P. The between-assay CV was 16.1% (E_2) and 8.9% (A). The within-assay CV averaged 8.7% for E_2 and 7.9% for A (6 analyses).

When different amounts of unlabelled steroids were added to the incubation medium, extracted and assayed, good agreement was found for both steroids between the amount added and that measured, as shown in Table IV.

It was further shown, for both steroids, that parallelism existed when different volumes of incubation medium were taken for RIA.

The recovery of radioactivity when ^{3}H -labelled steroids were added to samples and extracted with ether averaged 98% for both steroids.

Since the extraction of all three steroids measured was efficient and since the medium blank values (see above) were below or near the sensitivity limit of the standard curve (6.25 - 12.5 pg/600 µl), no correction was made for recovery or medium blanks.

Statistics

Values are generally expressed as mean $\pm$ SEM. Comparisons between two groups were made using Student's t-test and between several groups by analysis of variance followed by Student-Newman-Keul's multiple range test (Wolf 1968). A p-value less than 0.05 was considered significant.

RESULTS AND COMMENTS

The main results of papers I-IV are summarized and commented on in this section. More detailed considerations concerning the interpretation of the results will be given in "General discussion".

Steroidogenesis and hCG-responsiveness of isolated cells from preovulatory follicles (paper I).

It was demonstrated in paper I that both theca and granulosa cells from healthy, preovulatory follicles had the capacity to synthesize all three steroids as measured *in vitro*. The predominant steroid formed was P for granulosa cells and A for theca cells, while E_2 was formed in similar amounts by both cell types. From animal studies it has been claimed that steroidogenesis in the follicle is compartmentalized between the granulosa and the theca cells and that an interaction between these two cell types is required for the production of estrogens in the follicle (for refs see Armstrong and Dorrington 1977). Whether this theory is valid also in the human has been a matter of debate since different *in vitro* studies have yielded contradictory results. One reason for this may be that most earlier studies on the steroidogenic activity of human follicular cells were made without any knowledge as to the stage of follicular maturation or degree of atresia. Therefore, all follicles of the present study were carefully examined to assess that they were healthy and fully developed.

The findings of the present study demonstrate that, in the human preovulatory follicle, steroidogenesis is not rigidly compartmentalized between granulosa and theca cells.

In the present study it was further demonstrated that hCG stimulated cAMP formation in both granulosa and theca cells derived from preovulatory follicles. In addition, hCG stimulated the formation of P and A in the theca cells, but had no significant effect on these steroids in granulosa cells. There was, however, a tendency towards a stimulation of P in granulosa cells during 2 h incubations. This tendency has been further analysed in two additional experiments on granulosa cells from human preovulatory follicles by prolonging the incubation time to 4 h. It was then found (Table V) that hCG elicited a statistically highly significant increase in P formation. It can be seen further from Table V that the basal amounts of P formed by the granulosa cells from the two follicles were markedly different. This presumably reflects differences in previous exposure to LH *in vivo* both with respect

to the amount and the time. The steroidogenically most active follicle (follicle A) was markedly protruding from the ovary and exhibited an ovulation stigma, indicating that ovulation would have occured within few hours.

Table V. Release of progesterone into the incubation medium during a 4 h incubation period of human granulosa cells from two preovulatory follicles.

	Progesterone release (ng/mg protein)		
	control	hCG 100 IU/ml	significance
follicle A (diam: 25 mm)	907.5 ± 31.4	1270.4 ± 69.2	($p < 0.001$)
follicle B (diam: 23 mm)	221.4 ± 19.5	409.3 ± 37.4	($p < 0.01$)

The follicles were obtained from different patients. Follicle A was estimated to be within a few hours of ovulation. Values are given as means ± SEM.

It thus appears that human granulosa cells, as well as theca cells, from preovulatory follicles are capable of responding to hCG *in vitro* in terms of cAMP and P formation.

Effects of hCG and prostaglandins on isolated corpora lutea of different ages (paper II).

Prostaglandin E_2 (as well as LH and hCG) has been shown to stimulate P synthesis in human CL (Marsh and LeMaire 1974, Nakashima 1979), while the role of $PGF_{2\alpha}$ in regulating the function of the human CL has been a matter of controversy. Several attempts to demonstrate a luteolytic effect of $PGF_{2\alpha}$ *in vivo* in the human have so far been unsuccessful (e.g. Jewelewicz et al 1972, Kajanoja et al 1978). We have recently demonstrated that $PGF_{2\alpha}$ *in vitro* can counteract the stimulatory effect of hCG on cAMP formation in human CL of mid-luteal phase, indicating a role for $PGF_{2\alpha}$ in the luteolytic process of the human (Hamberger et al 1979).

In the present study (paper II) the effects of $PGF_{2\alpha}$ and of PGE_2 on the formation of cAMP and P were investigated, both alone and in combination with hCG, in human CL of different ages. It was clearly demonstrated that $PGF_{2\alpha}$ significantly counteracted the stimulatory effect of hCG on both cAMP and P formation in CL of mid-luteal phase, whereas in young and old CL, $PGF_{2\alpha}$ did not interfere with the effects of hCG. PGE_2, on the other hand,

did not significantly alter the effect of hCG in CL of mid-luteal phase, while in young CL PGE_2 potentiated the stimulatory effect of hCG on cAMP formation. Furthermore, PGE_2, per se, caused a statistically significant increase in cAMP formation in young CL. In two additional experiments we also tested the effects of PGE_2 on P formation in young CL. As seen from Table VI, PGE_2 caused a slight, but insignificant increase in P production. The possibility that PGE_2 stimulates the formation of other steroids more markedly remains to be settled.

Table VI. Influence of PGE_2 (1 µg/ml) alone and in combination with hCG (100 IU/ml) on progesterone release into the incubation medium during a 2 h incubation period of human CL of early luteal phase.

Treatment	Progesterone release (% of control value)
control	100 ± 11.1
hCG 100 IU/ml	144.4 ± 34.4 [1]
PGE_2 1 µg/ml	128.8 ± 34.9 [1]
hCG 100 IU/ml + PGE_2 1 µg/ml	141.1 ± 48.6 [1]

Results from two CL have been pooled and are expressed as means ± SEM.

1/ NS vs control

It can be concluded from this particular in vitro study that, while hCG has luteotropic effects in all CL up to 13 days of age, the effects of PGE_2 and $PGF_{2\alpha}$ may be qualitatively opposite, depending on the age of the CL. In young CL PGE_2 may have a luteotropic effect and in CL of mid-luteal phase $PGF_{2\alpha}$ is luteolytic.

Steroid formation and effects of hCG in postmenopausal ovarian stromal tissue (paper III).

The ovarian stroma has been suggested to be a distinct gland of internal secretion within the ovary (Rice and Savard 1966). Several in vitro studies have shown that this compartment, in both the fertile and the postmenopausal ovary, has the capacity to produce androgenic steroids (e.g. Rice and Savard 1966, Mattingly and Huang 1969). The stroma of postmenopausal ovaries has been claimed to be inactive in the aromatization of androgens to estrogens (Mattingly and Huang 1969). This concept is contradicted by the results of

this study (paper III). It was found that specimens of "normal" atrophic stroma had the capacity to produce measurable amounts of E_2, as well as P and A, *in vitro*. Furthermore, hyperplastic stroma from postmenopausal ovaries produced significantly higher amounts of E_2 *in vitro* than did atropic stroma. Also the production of A was considerably higher in ovaries with stromal hyperplasia, while P was formed in small and similar amounts by both stromal types.

It was further found that hCG caused a statistically significant increase in cAMP formation in hyperplastic stroma but not in atrophic stroma. These results indicate a preserved responsiveness to gonadotropin in postmenopausal ovaries with stromal hyperplasia, although the steroid formation *in vitro* was not significantly altered by hCG during a 4 h incubation period.

Thus it appears that the endocrine activity in postmenopausal ovarian stroma varies considerably from a relatively low capacity for steroid production in atrophic stroma to a considerable degree of steroid formation and a clearcut aromatizing capacity in hyperplastic stroma.

Steroid production and effects of hCG in postmenopausal ovarian hilus cells (paper IV).

Since the ovarian hilus cells are irregularly distributed as small nests in the hilar region, these cells are difficult to identify morphologically. Therefore direct *in vitro* studies on the endocrine function of the hilus cells are lacking. There are, however, some indications that these cells are steroidogenically active: considerable activity in the hilus cells of certain oxidative enzymes generally associated with active steroidogenesis (Scully and Cohen 1964), and association between hyperplasia of the hilus cells and excess androgen production (Dennefors et al 1979).

In the present study an attempt was made to identify hilus cells in specimens from the hilar tissue of postmenopausal ovaries and to study their endocrine activity *in vitro*. Strips of hilus tissue were carefully examined histologically by serial sectioning and typical hilus cell clusters were identified. When these tissue specimens were incubated for 4 h, measureable amounts of all three steroids were found. The pattern of steroidogenesis was similar to that of normal postmenopausal stroma with A being the predominant steroid formed. The absolute amounts of steroids produced by the hilus tissue were, however, approx. 2-fold higher than those produced by normal stroma when related to the protein content of the specimens (compare Fig 2, paper IV with Fig 3, paper III). Since the incubated specimens of hilus cells also contained protein from other, steroidogenically inactive struc-

tures, this difference would probably be even more pronounced had the steroid formation been related to the number of hilus and stromal cells.

It was concluded that the hilus cells in human postmenopausal ovaries are steroidogenically active and that they may form a separate functional compartment of the ovary.

It was further shown in paper IV that hCG elicited a statistically significant increase of cAMP formation after 30 min incubation and of P and E_2 formation after 4 h incubation. It thus appears that the hilus cells from postmenopausal ovaries have a preserved responsiveness to hCG despite the fact that these cells are exposed continuously to elevated, endogenous LH-levels in the circulation.

GENERAL DISCUSSION

In this section the main findings of papers I - IV will be discussed in relation to relevant data from the literature. The emphasis will be on human studies with some necessary comparisons with animal experiments. The considerations will be given separately to each of the different compartments of the ovary, although it must be kept in mind that these compartments are intimately linked to each other both morphologically and functionally. Recent studies have shown that the various cell types of the human ovary can interact with each other to modify steroid production in vitro (McNatty et al 1980[a], Batta et al 1980).

In the last part of this section some possible clinical implications of the present and other related studies will be given.

Follicle

It is well established that almost all (99.9%) human follicles degenerate at various stages of development (Byskov 1978), most of them during early antral development (McNatty 1981). When studying human follicular function it is thus of crucial importance to determine whether a follicle is growing (i.e. has the potential for further development) or is atretic. Based on studies of human antral follicles McNatty and coworkers (1979[b]) have suggested a series of parameters by which the degree of growth or atresia can be assessed. Growing follicles are characterized by: 1) more than 50% of the optimal number of granulosa cells corresponding to the follicular diameter, 2) high concentrations of E_2 (> 1000 ng/ml) in the follicular fluid or granulosa cells with the capacity to increase E_2 synthesis in response to FSH, and, 3) a healthy looking germinal-vesicle stage oocyte. Atretic follicles are characterized by a relatively low number of granulosa cells (less than 50% of the full complement of granulosa cells for their respective diameter), by low concentrations of E_2 in the follicular fluid and by morphological signs of degeneration of the oocyte (McNatty et al 1979[b]). Other studies have confirmed the validity of these criteria for assessing a follicle as growing or atretic (McNatty 1979[a], Hillier et al 1980).

Atretic follicles: It has been shown that isolated cells from human atretic follicles produce mainly A and only small amounts of E_2 and P (McNatty et al 1979[a], Dennefors and Nilsson, to be published). Therefore, these follicles have an androgen-enriched follicular fluid in which the mean level of A

is generally more than 30-fold higher than that of E_2 (McNatty et al 1979[b]). In a recent study on intact human ovarian follicles explanted at term pregnancy, we found that these follicles, which all showed histological signs of atresia, secreted large amounts of A and small amounts of P and E_2 during short term incubations (Dennefors and Nilsson 1981).

Growing follicles: Although the fate of most follicles is to undergo atresia, at least one follicle is selected for further development and ovulation in every normal cycle. The mechanism of action underlying this selection is still an enigma, although it appears that the hormonal environment within the follicle is one key factor in determining its subsequent development. Since only follicles with an "estrogenic" microenvironment have the potential for further growth it has been suggested that the development of a functional aromatase enzyme system in the follicle during early follicular growth is of crucial importance for continuation of growth (McNatty 1981).

When a certain follicle has been selected for further development, it enlarges and accumulates large numbers of granulosa cells, substantial theca mass and increasing amounts of follicular fluid. As a consequence, the follicle becomes more active steroidogenically.

During the early and mid-follicular phases, the ovary secretes increasing amounts of E_2 and, to a lesser extent, A. The thecal tissue of growing, not fully developed follicles has been found to produce mainly A and small amounts of estrogens and P *in vitro* (Mc Natty et al 1979[a]). Consequently the granulosa cells have been claimed to be the major source of estrogen production in these follicles. Moon and coworkers (1978) found that human granulosa cells of growing follicles produced little or no estrogen in culture unless testosterone was added as a substrate. On the other hand, McNatty et al (1979[a]), using a similar experimental system, reported that granulosa cells from growing follicles cultured without precursors produced considerable amounts of E_2 and small amounts of A and P. Although this discrepancy is difficult to explain, it may well be due to differing amounts of precursors present in the serum-enriched culture media, or to varying stages of development of the follicles studied. Nevertheless, the results from these two studies concur in that human granulosa cells from growing follicles have an aromatizing capacity. This has been confirmed by Erickson and coworkers (1979) who also demonstrated that granulosa cells from human follicles achieve a functional aromatase system when the follicle has reached a certain stage of development (6-8 mm in diameter). Furthermore, numerous studies from various animal species on isolated granulosa cells in culture

have provided direct biochemical data that granulosa cells can convert androgens to estrogens but have low capacity to synthesize androgens from pregnenolone or progesterone (for refs see Richards 1980). Taken together with the findings that the theca is the major source of androgens, these data support the "two-cell" hypothesis for ovarian estrogen secretion originally proposed by Falck (1959) and subsequently modified by Armstrong and Dorrington (1977). This hypothesis states that androgens produced by the theca cells diffuse to and are aromatized by the granulosa cells.

The steroidogenesis in the growing follicle is regulated by both LH and FSH. It has been shown that human theca cells are responsive to LH/hCG, but not to FSH, in terms of cAMP and steroid formation (Tsang et al 1979, McNatty et al 1980[b], Dennefors and Nilsson, to be published), and receptors for LH/hCG have been demonstrated on the theca cells of pig and rat follicles (Rajaniemi and Vanha Perttula 1972, Channing and Kammerman 1974). FSH, but not LH/hCG, has been found to stimulate the aromatase activity of human granulosa cells in vitro (Moon et al 1978, Erickson et al 1979, Hillier et al 1980) and, further, FSH receptors have been located on the granulosa cells in the rat (Midgley et al 1974, Richards et al 1976). Thus, during follicular development, FSH plays an important role in stimulating the aromatization of androgens by the granulosa cells, thereby sustaining a follicular "estrogenic" microenvironment, which, according to the above mentioned theory, is a prerequisite for further development into a preovulatory follicle.

Preovulatory follicles: When the follicle reaches its final phase of maturation it has accumulated most of its granulosa and theca cells. However, the follicle continues to grow during the preovulatory period and this enlargement (from approx. 12 to 25-30 mm in diameter) is due almost entirely to accumulation of follicular fluid (McNatty 1981). This large preovulatory follicle is steroidogenically more active than any other follicle in the ovary and is the major source of estrogen production (Baird and Fraser 1974, Richards 1980).

There have been relatively few studies on the steroidogenic capacity of the theca cells from human preovulatory follicles, probably due to difficulties in obtaining a theca cell preparation free from contaminating granulosa cells. McNatty and coworkers (1979[a]) reported, in a study on a small number of human preovulatory follicles, that the thecal tissue from these follicles secreted mainly A, but also some P and E_2, in culture. Evidence that the theca is the major follicular source of androgens has also been

obtained from studies in different animal species (for refs see Richards 1980). The results of the present study (paper I) demonstrate that acutely incubated theca cells from preovulatory follicles produce A as the dominant steroid and that, in addition, considerable amounts of E_2, and some P, are produced as well. This pattern of steroidogenesis in the theca cells of preovulatory follicles is similar to that of less developed follicles (see above), but the relative amount of E_2 formed is higher when the follicle is preovulatory.

The preovulatory granulosa cells of the present study had the capacity to produce all three steroids measured in vitro. The major steroid formed was P, but considerable amounts of E_2 were also produced. These data are in agreement with the results of McNatty and coworkers (1979[a]) who found a similar pattern of steroidogenesis in cultured granulosa cells from large, human follicles.

The validity of the "two cell" theory for estrogen secretion in the preovulatory follicle has been a matter of debate. For instance, Channing and Coudert (1976) reported that estrogen secretion was maintained after the aspiration of granulosa cells from monkey preovulatory follicles in vivo, indicating that the theca itself is able to synthesize E_2. The results of the present study strongly indicate that steroidogenesis in the human preovulatory follicle is not rigidly compartmentalized, but they do not completely oppose the "two-cell" theory. Since the patterns of steroidogenesis in the two cell types of the preovulatory follicle are different from one another, a certain degree of collaboration between the two cell types in modifying the steroid output of the follicle may exist in vivo. This concept is further strengthened by the finding of McNatty and coworkers (1980[a]) that recombination of isolated granulosa and theca cells from large human follicles caused a synergistic production of P and E_2 after 48 h of co-culture (for E_2 only in the presence of LH and FSH).

It thus seems clear, that when the follicle develops into a preovulatory structure, its granulosa cells change steroid production from predominantly E_2 to predominantly P. This shift in granulosa cell steroidogenesis is reflected in changes in antral fluid steroid concentrations. During the late follicular phase, the levels of estrogens in antral fluid decline, and those of P strongly increase (McNatty 1978). These changes in granulosa cell function are most likely regulated by endogenous gonadotropins.

In paper I it was shown that hCG stimulated cAMP and steroid formation in preovulatory theca cells, and this is in agreement with the few other human studies which have reported a similar stimulatory effect of LH/hCG on

theca cells from follicles of various stages of development (Tsang et al 1979, McNatty 1980[b]). Furthermore, receptors for LH/hCG have been demonstrated on the theca cells of pig and rat follicles (Rajaniemi and Vanha-Perttula 1972 , Channing and Kammerman 1974). FSH, on the other hand, has been found to have no effect on human theca cells as judged by cAMP and steroid formation (Tsang et al 1979). Thus, it may be concluded that steroidogenesis in human theca cells of mature follicles is regulated exclusively by LH.

As mentioned earlier, granulosa cells possess receptors for FSH and this hormone has been found to stimulate the aromatase enzyme system in human granulosa cells from growing follicles. LH/hCG, on the other hand, has no effects on these immature granulosa cells as judged from cAMP and steroid formation (Moon et al 1978, Tsang et al 1979, Hillier et al 1980). This coincides with the findings in the rat that functional receptors for LH/hCG are located on granulosa cells from large, preovulatory follicles but not from follicles in earlier stages of development (Zeleznik et al 1974). It has recently been shown that follicular maturation in the human is associated with an increase in the number of LH/hCG receptors on the granulosa cells (Rajaniemi et al 1981). In paper I, a clearcut stimulation of cAMP formation by hCG was demonstrated in the granulosa cells from preovulatory follicles and, in two additional experiments, a significant increase in P production in response to hCG was found (Table V, p.29). Although this acute stimulatory effect of hCG on granulosa cells from preovulatory follicles is in agreement with the above mentioned receptor findings, it has never previously been demonstrated in the human. This could be due to the fact that only granulosa cells from truly preovulatory follicles can respond to LH/hCG. Prolonged influence of LH in vivo has been reported to induce a block in the formation of A in both granulosa and theca cells from rat preovulatory follicles, thereby causing a shift in steroidogenesis of the whole follicle from E_2 towards P formation (Hamberger et al 1978). The above findings in isolated human granulosa cells might indicate the existence of a similar mechanism in the primate.

Thus, when the follicle has reached full maturation its granulosa cells have achieved a responsiveness to LH/hCG, probably as a result of a stimulatory effect on these cells by increasing follicular levels of FSH and E_2 (Zeleznik et al 1974, Channing 1975). This achieved responsiveness to LH of the granulosa cells concomitant with the endogenous LH surge appears to have important implications: 1) inhibition of further granulosa cell proliferation (Delforge et al 1972, Rao et al 1978) and 2) shift from estrogen to progesterone secretion and luteinization.

After ovulation, the preovulatory follicle is transformed into another ovarian compartment, the CL. However, the ultimate fate of all the atretic follicles is less clear but, sooner or later, they disappear into, or possibly form a part of, the ovarian stroma (Mossman et al 1964).

Corpus luteum

When the CL is formed the luteinized granulosa cells, which form the major proportion of the CL, are producing considerable amounts of P. This is the dominant steroid secreted by the ovary during the luteal phase. Estrogens also are produced by the human CL, and this is a unique feature for the primate CL (Savard et al 1965, Baird and Fraser 1974, Challis et al 1976). *In vivo* studies have shown that approx. 95% of the production of E_2 by the human ovary during the luteal phase originates from the CL (Baird and Fraser 1974). From direct studies on the concentration of steroids in the human CL (Challis et al 1976, Patwardhan and Lanthier 1980) and from short term incubations (Nakashima 1979, Dennefors, unpublished observations) it can be estimated on a molar basis that the total production of P is approx. 20-50 fold higher than that of E_2 during the life span of the CL. The results of the present study (paper II) show that the amounts of P produced per mg protein by newly formed CL are 3-4 fold lower than those formed by mid-luteal phase CL, and of the same magnitude as those produced by late luteal phase CL. Since these *in vitro* data are in good agreement with the dynamics in P secretion by the CL *in vivo*, they contradict the concept that it is principally the increase in CL mass which accounts for the progressive increase in P secretion during the early and mid-luteal phase (Armstrong and Black 1966). Furthermore, the production of E_2 by the CL appears to change during the luteal phase, with the highest amounts of E_2 formed in mid-luteal phase (Swanston et al 1977).

The life span and function of the CL appear to be regulated by a complex system of intrinsic and extrinsic stimulatory (luteotropic) and inhibitory (luteolytic) factors. Corpora lutea induced in hypophysectomized women by treatment with FSH and LH will function for about 3-4 days without any further treatment (Vande Wiele et al 1970). This finding indicates that, although the human CL is autonomous to a certain extent, it needs continuous support of LH for its normal function. LH, or hCG during pregnancy, appears to be the major extrinsic luteotropin in the human, and has been shown to stimulate both cAMP (Marsh and LeMaire 1972) and P production (Rice et al 1964, Hanson et al 1971) in the human CL. Also in the present study, a stimulatory effect of hCG on both cAMP and P formation was noted in CL up

to 13 days of age (paper II). The responsiveness to hCG was most pronounced in CL of the mid-luteal phase. In an earlier study, using the same in vitro system, we have shown that the sensitivity to hCG of human CL declines at the time around the menstruation (14 - 15 days after ovulation) (Hamberger et al 1979). This apparently rather abrupt decline in gonadotropin sensitivity may be due, at least partly, to a loss of gonadotropin receptors (Conti et al 1977, McNeilly et al 1980).

Prolactin is an important luteotropic hormone in several animal species e.g. the rat (for refs see Rothchild 1981). McNatty and coworkers (1974) have reported that the production of P by human luteinized granulosa cells in culture requires low concentrations of prolactin, indicating that this hormone has either a true or a permissive luteotropic effect also in the human. Hyperprolactinemia, on the other hand, is associated with short luteal phase and reduction in P secretion in the human (Schneider et al 1977). The precise role of prolactin in regulating the human CL is unclear to date and has not been investigated in the present study.

In addition to these extrinsic luteotropins, hormones produced within the CL may possess luteotropic effects. One such hormone is PGE_2, which is synthesized by the human CL (Challis et al 1976) and which has been shown to stimulate cAMP and P formation in human CL of non classified ages in vitro (Marsh and LeMaire 1974). In the present study it was found that PGE_2 stimulated cAMP formation in young CL but not in CL of mid-luteal phase. In two additional experiments performed on young CL a weak but non significant increase in P production was found (Table VI, p. 30). Although not conclusive, these data indicate that PGE_2 might have a luteotropic effect in the newly formed human CL.

Interestingly, P itself has been suggested as an intrinsic luteotropin by Rothchild (1981). The strongest support for the suggestion that P promotes its own secretion is the direct relationship between intraluteal P concentration and the rate of P secretion, which is in contrast to the lack of relation between P secretion by the CL of the cycle and that of its presumed extrinsic stimulus (LH) (Rothchild 1981). The hypothesis that P stimulates its own secretion may explain why the CL can function autonomously for a limited time (see above).

There is overwhelming evidence that uterine $PGF_{2\alpha}$ is the physiological luteolysin in the sheep (for review see Horton and Poyser 1976). In many other species (e.g. rabbits, rats, cows, pigs, horses) $PGF_{2\alpha}$ can induce luteolysis (Horton and Poyser 1976), although a uterine production of $PGF_{2\alpha}$ is not of essential importance in all species. It is well known, however,

that $PGF_{2\alpha}$ is produced by the CL, both in animals (Demers et al 1973) and in the human (Challis et al 1976), indicating that $PGF_{2\alpha}$ may be an intrinsic luteolytic factor. There is so far no conclusive evidence for a luteolytic effect of $PGF_{2\alpha}$ *in vivo* in the human, but this could solely be due to the inability to reach adequate local ovarian concentrations of $PGF_{2\alpha}$ when the compound is given systemically. By injecting very high doses of $PGF_{2\alpha}$ via laparoscopy directly into the human CL, Korda and coworkers (1975) could demonstrate a profound fall in plasma P levels which was followed by a menstrual like bleeding. Similar experiments have recently been performed in the rhesus monkey using continuous infusions of lower doses of $PGF_{2\alpha}$ (Auletta et al 1981). It was then found that intraluteal $PGF_{2\alpha}$ induced functional luteolysis in this species.

In 1975, McNatty and coworkers demonstrated a "luteolytic" effect of $PGF_{2\alpha}$ *in vitro* on human luteinized granulosa cells in culture. Four years later we showed that $PGF_{2\alpha}$ *in vitro* could counteract the stimulatory effect of hCG on cAMP formation in human CL of mid-luteal phase (Hamberger et al 1979). In the present study on isolated human CL of well defined ages, $PGF_{2\alpha}$ significantly inhibited the stimulatory effect of hCG on both cAMP and P formation in mid-luteal phase CL. Although $PGF_{2\alpha}$ had no effect per se, these data strongly indicate that $PGF_{2\alpha}$ can cause luteolysis by possessing an antigonadotropic effect. The concept that $PGF_{2\alpha}$ is a physiologic luteolytic hormone in the human is strengthened by the finding that human CL possess receptors for $PGF_{2\alpha}$ (Powell et al 1974), and that these receptors have many characteristics similar to those found in bovine CL (Rao et al 1977), where $PGF_{2\alpha}$ undoubtedly is luteolytic.

The reason why $PGF_{2\alpha}$ has an antigonadotropic effect only in CL of the mid-luteal phase, and not in young or old CL, has been explored in another series of experiments in our laboratory. In the newly formed PMSG induced CL of the rat, blood flow is extremely high (Damber et al 1981). The maximally dilated vessels are devoid of innervation at this early stage. When the rat CL is five to eight days of age, a norepinephrine (NE) innervation of the vessels appears concomitant with a slight decrease in CL blood flow, probably due to an increased vascular tonus, and with the appearance of a maximal interaction between LH/hCG and $PGF_{2\alpha}$ (Hamberger et al 1980). A similar situation appears to exist in women. These findings indicate that the endogenous amounts of NE in the CL are of importance in modifying the effects of $PGF_{2\alpha}$. This hypothesis was tested in the human by adding NE to incubated specimens of young CL. It was then found that $PGF_{2\alpha}$ counteracted the stimulatory effect of hCG on cAMP formation also in these young CL. Furthermore,

when reserpine was added to incubated mid-luteal phase CL (to deplete the specimens of cathecolamines), the antigonadotropic effect of $PGF_{2\alpha}$ was absent (Hamberger et al 1980). These data thus suggest that vascular innervation is a prerequisite for PG-induced luteolysis in the human. If this theory proves correct it can also explain why attempts to demonstrate a luteolytic effect of $PGF_{2\alpha}$ in dispersed human luteal cells have been unsuccessful (e.g. Richardson and Masson 1980). Tissue integrity seems to be of essential importance, at least when it concerns the human CL. The fact that $PGF_{2\alpha}$ exerted its luteolytic effect in an in vitro system indicates that the compound does not act directly on blood flow. This concept is in agreement with recent animal data (Pang and Behrman 1979, Damber et al 1981).

Estrogens have been implicated in the luteolytic process in the human, since it has been shown that administration of estrogens in vivo to women in the postovulatory phase of the normal cycle can cause luteolysis (Hoffman 1960, Johansson and Gemzell 1971, Gore et al 1973), and that E_2 in vitro can inhibit the stimulatory effect of hCG on P synthesis in isolated human luteal cells (Williams et al 1979). It is at present unclear whether E_2 has a direct luteolytic effect on the human CL or acts indirectly via intraluteal $PGF_{2\alpha}$, as has been suggested by Auletta and coworkers (1978) in their report on primate experiments. In a recent in vitro study on dispersed human luteal cells the addition of indomethacin did not prevent the inhibitory effect of E_2 on hCG stimulated P accumulation, although the accumulation of $PGF_{2\alpha}$ was markedly reduced (Thibier et al 1980).

Ovarian stroma

The biochemistry of the stromal elements of the ovary has not been studied to the same extent as that of the follicle or the CL. As mentioned earlier, Rice and Savard (1966) were the first to report that the stroma from human ovaries, obtained at different phases of the menstrual cycle or during pregnancy, is capable of producing androgenic steroids in vitro. More recently, McNatty and coworkers (1979[a]) demonstrated that the ovarian stroma from women at different phases of the menstrual cycle had the capacity to produce P, androgens and estrogens during tissue culture. The relative amounts of steroids produced by the stroma varied with the phase of the menstrual cycle, the highest amounts being found during the late follicular phase, probably due to a previous exposure to gonadotropins in vivo. Androstenedione was the major steroid formed in vitro at all stages of the menstrual cycle, despite P being formed in similar amounts to A during the late follicular phase. The stromal production of estrogens in vitro was low at all phases

of the cycle.

Mattingly and Huang (1969) reported that the stroma from postmenopausal ovaries also can produce androgens *in vitro*. Since only trace amounts of estrogens were formed in their study, these authors concluded that the postmenopausal stroma is inactive in the aromatization of androgens to estrogens. However, later *in vivo* studies have shown significantly higher amounts of E_2 in ovarian vein blood when compared to peripheral vein blood in postmenopausal women (Judd et al 1974, Greenblatt et al 1976), indicating a certain, although probably limited, aromatazing capacity of the postmenopausal ovary. These latter findings are more in accordance with the results of the present study which show that stromal tissue from postmenopausal ovaries can form E_2 *in vitro*.

Around and after the menopause the ovarian stroma often shows signs of moderate to marked proliferation, with the highest incidence (36%) occurring between 56 - 70 years of age (Boss et al 1965). Therefore, it has been hypothesized that the high endogenous gonadotropins in the postmenopausal period may induce stromal hyperplasia (Boss et al 1965). This hypothesis is now further supported by the finding from the present study that only specimens with clear stromal hyperplasia can respond to hCG in terms of increased cAMP formation, demonstrating a preserved gonadotropic responsiveness in these ovaries. Hyperplastic stroma reacted differently from atrophic stroma in yet another way. The basal production of A and E_2 was more than two-fold higher in hyperplastic than in atrophic stroma, this higher production of E_2 by hyperplastic stroma is especially interesting in view of the possible association between stromal hyperplasia and endometrial carcinoma, as discussed below. Only cases with clear signs of stromal hyperplasia (as defined in paper III) were included in the group of hyperplasia. Thus the separation of the hyperplastic group from the atrophic group was morphologically distinct (Fig 1 and 2, paper III). However, it must be emphasized that stromal hyperplasia is a condition where the borderline between the normal and pathologic states cannot be readily distinguished. The results of the present study indicate that the cell types within the hyperplastic stroma may be of greater significance for functional activity than the actual quantity of hyperplastic stroma. There are also indications that the process of hyperplasia is reversible (Boss et al 1965).

It thus appears from the present and other studies that the stroma of the human ovary is a distinct functional compartment producing mainly androgens. After the menopause, when the stroma forms the major part of the ovary, this compartment may become more or less hyperplastic, probably due to

gonadotropic stimulation, with consequently increased secretion of A and E_2 by the ovary. The variability of the endocrine activity in the postmenopausal ovary has recently been demonstrated by Longcope et al (1980), who measured the concentration of steroids in ovarian arterial and venous blood in a large number of postmenopausal women. The steroid levels varied markedly between different women, but in 30% of the patients considerably higher levels of E_2 and A were found in venous as compared to arterial blood. This increased ovarian steroid production in some of the patients could have been caused by stromal hyperplasia in the ovaries. Another possible source of increased steroid production could be the hilus tissue.

Hilus cells

Nests of hilus cells can be found microscopically in the ovarian hilus of probably all adult ovaries, but the number of hilus cells varies considerably between different women (Sternberg 1949). These cells are particularly prominent during pregnancy, and during and after the menopause, probably due to influence of gonadotropins (Sternberg 1949, Boss et al 1965). Histochemical studies have demonstrated strong activity of certain oxidative enzymes, associated with active steroidogenesis, in the hilus cells (Scully and Cohen 1964). Since the hilus cells are morphologically identical with testicular Leydig cells, and since hilus cell hyperplasia and tumors are often associated with virilization, these cells have been claimed to secrete androgens (Sternberg 1949).

In the present study (paper IV) specimens from the hilar tissue of postmenopausal ovaries were isolated and found to contain typical hilus cells. When these specimens were incubated, a similar pattern of steroidogenesis as in atrophic stroma was demonstrated, with A being the major steroid formed. These data therefore confirm that the hilus cells from postmenopausal ovaries are steroidogenically active producing mainly androgens. Since the hilus cells are embryologically and histologically different from the stroma cells, it is reasonable to suggest that the hilus cells form an additional functional compartment of the human ovary.

In the present study the amounts of steroids formed *in vitro* were considerably higher in hilus cells than in stromal cells when related to tissue protein content (p. 31). It must be remembered, however, that the total mass of stroma is much higher than that of hilus cells in the postmenopausal ovary. Therefore, the hilus cells probably contribute to only a small portion of the total steroid output of the postmenopausal ovary in the physiological situation.

Earlier studies have shown that the hilus cells of both fertile and postmenopausal ovaries can respond to hCG *in vivo* as judged by morphological changes of the cells (Sternberg et al 1953) and histochemical evidence of stimulation (Poliak et al 1968). This statement is confirmed more directly by the results of the present *in vitro* study, which clearly demonstrate that the hilus cells can respond to hCG with increased cAMP and steroid formation. Of particular interest is the marked increase in E_2 production in view of the high endogenous levels of LH during the postmenopausal period. The hilus cells could be a potential source of estrogen production in the postmenopausal ovary. In women with a large number of hilus cells, this compartment may be of both physiological and clinical importance.

Clinical implications

In paper I it was demonstrated that human preovulatory follicles may reach a diameter up to at least 25-30 mm. This implies that so called "*follicular cysts*" of such a size should not be resected routinely during laparotomy of young women, since these "cysts" are most likely normal preovulatory follicles. This statement does not, of course, apply to small ovarian cysts with suspicious signs of neoplasia or endometriosis.

In the previous section a sequence of hormonal changes crucial for normal follicular development has been described. In short, this development requires a certain "threshold" level of FSH during the early and mid-follicular phases to stimulate the granulosa cells for conversion of androgens to estrogens, thereby generating an estrogen-enriched environment within the follicle. When the follicle is fully mature (preovulatory) both granulosa and theca cells are responsive to LH. The preovulatory levels of LH arrest further granulosa cell proliferation and induce luteinization of the follicle. Based on this precise sequence of FSH and LH release *in vivo*, some important implications concerning the *treatment of anovulatory women with exogenous gonadotropins* can be drawn. These gonadotropins should ideally be given in a schedule imitating the normal *in vivo* situation and, therefore, daily estimations of estrogens in urine, or preferably blood, are necessary. In the early and mid-follicular phases exogenous FSH must be given in amounts great enough to stimulate the aromatizing capacity of the granulosa cells, thereby avoiding accumulation of large concentrations of androgens in the follicular fluid. Another crucial point is to give LH or hCG at exactly the right time. If LH is given too early (i.e. before the granulosa cells are mature enough to respond fully to LH), LH blocks granulosa cell mitoses and

stimulates the theca cells to increased androgen production, the latter leading to increasing amounts of androgens in the follicular fluid. Since the granulosa cells are no longer capable of converting these androgens to estrogens, an androgen-enriched environment within the follicle will be generated, with the consequence that the follicle goes into irreversible atresia. Such a paradoxical action of hCG, when administered prematurely during the late follicular phase, has recently been demonstrated in monkeys (Williams and Hodgen 1980).

In the last 10 years several studies have clarified some of the pathophysiologic events of the polycystic ovary syndrome (PCO), although its primary causes have not been defined. This syndrome is characterized by the presence of multiple, medium sized ovarian follicles (2-6 mm in diameter). It has been shown that these follicles contain high concentrations of A and very small amounts of E_2 or estrone in their follicular fluid, indicating a deficient aromatase enzyme activity in the granulosa cells (Short and London 1961). However, since the normal growing human follicle does not aquire an effective aromatase system until it has reached a diameter of 6-8 mm (Erickson et al 1979), these findings in the PCO follicles are actually not abnormal. Studies of isolated granulosa and theca cells derived from follicles of normal and PCO ovaries have shown, that these cells, from both types of ovaries, have a similar capacity to produce steroids in vitro, and further, that the granulosa cells from PCO follicles can respond to FSH with increased estrogen secretion both in vivo and in vitro (Erickson et al 1979, Wilson et al 1979). These findings suggest that the absence of aromatase activity in the granulosa cells from patients with PCO is caused by an inappropriate stimulation of the ovaries by FSH, and not by an inherent defect in the follicular cells (Erickson et al 1979).

The stromal compartment also has been suggested to be involved in the pathophysiology of PCO (Yen 1980). Rice and Savard (1966) studied the in vitro steroid formation in stroma from patients with PCO comparing it to normal stroma from patients of various phases of the cycle. They found that the spectrum of steroids formed in vitro was strikingly similar between the two groups. The absolute amounts of steroids formed (as judged from incorporation of radiolabelled acetate) were, however, considerably higher in the stroma from PCO patients than in normal, mid-follicular phase stroma, and were of the same magnitude as the amounts formed by normal stroma from the late follicular phase. Based on these findings, the authors suggested that the high amounts of steroids (mainly androgens) produced by the stroma in

patients with PCO are merely an effect of persistent and high levels of endogenous gonadotropins (Rice and Savard 1966).

Although the causes and pathogenesis of PCO are still obscure, it appears that the endocrine changes noted in the ovaries of this disease are the results of an abnormal gonadotropic influence, and are not caused by a primary defect in ovarian steroidogenesis.

Numerous publications during the past three decades have discussed the alleged relationship of ovarian stromal hyperplasia to endometrial carcinoma. A significantly higher percentage of stromal hyperplasia in the ovaries of women with this type of cancer as compared to postmortem control specimens has been reported by many groups (e.g. Woll et al 1948, Novak and Mohler 1953). However, investigations comparing ovaries obtained at operation with postmortem control ovaries can be critized. Other authors, which have used surgical specimens as controls, have not found any consistent changes in the ovaries of patients with endometrial hyperplasia or endometrial carcinoma (e.g. Roddick and Greene 1957, Bigelow 1958).

It is generally agreed that prolonged, unopposed administration of exogenous estrogens can produce endometrial hyperplasia and cancer. Whether or not endogenous estrogens are involved in the pathogenesis of endometrial carcinoma is less clear. Some studies in postmenopausal women with endometrial cancer or hyperplasia have reported an elevated urinary estrogen excretion when compared to postmenopausal control subjects (Rome et al 1977, Reti et al 1978), while in other studies no differences in estrogen excretion were found between these two groups (Charles et al 1965, Procopé 1968). Both Judd and coworkers (1976) and Calanog and coworkers (1977) measured serum levels of E_2 and estrone in postmenopausal women with and without endometrial cancer. No statistically significant differences were found between the mean values of E_2 and estrone in these two studies. It is, however, noteworthy that the individual serum levels of E_2 and estrone in some of the cancer patients were clearly higher than in the control groups, in both studies. Furthermore, in Calanog's study significantly higher serum levels of A were found in the cancer group when compared to the control group. It is well documented that circulating A can be converted to estrone in peripheral tissues, such as fat and muscle, and this conversion rate has been claimed to increase with age (Hemsell et al 1974). In addition, several *in vivo* investigations have shown that the peripheral conversion of A to estrone is significantly higher (approx. two-fold) in patients with endometrial cancer than in non-cancer controls (Hausknecht and Gusberg 1973,

Siiteri and McDonald 1973, Calanog et al 1977). This difference has been claimed, however, to be due mainly to a higher incidence of obesity in the cancer group (Rizkallah et al 1975). On the other hand, Schindler et al (1972) and Forney et al (1981) demonstrated that the peripheral conversion of A to estrone by human fat tissue *in vitro* was significantly higher in patients with endometrial cancer than in healthy controls.

The results of the present study (paper III), demonstrating an increased androgen and estrogen production in postmenopausal ovaries with stromal hyperplasia, contribute to the discussion of the causual relationship between stromal hyperplasia, increased estrogen production and endometrial cancer. Based on the above mentioned studies and the present study it is suggested that ovaries with stromal hyperplasia may play a role in the pathogenesis of endometrial cancer in some postmenopausal women, by producing substantial amounts of estrogens, both directly and indirectly via peripheral conversion of ovarian androgens. This increased production of estrogens *in vivo* is probably more pronounced if the patient has stromal hyperplasia and is obese, a combination which is not unusual according to Boss and coworkers (1965).

Ovarian stromal hyperplasia can also have other clinical implications. Boss and coworkers (1965) found a correlation between moderate to marked stromal hyperplasia and varying degrees of *virilization* in postmenopausal women. This correlation is especially strong when abundant theca-lutein-like cells occur in the hyperplastic stroma (hyperthecosis), and such ovaries have been shown to produce several fold more androgens than normal ovaries in both postmenopausal and younger women with signs of virilization (Aiman et al 1978, Braithwaite et al 1978). The results of the present study support the concept that stromal hyperplasia can cause hirsutism and varying degrees of virilization in postmenopausal women, by increased ovarian androgen production.

Boss and coworkers (1965) have observed a strong positive correlation between the degree of stromal hyperplasia and the number of hilus cells in postmenopausal women. Since, in the present study, the hilus cells were found to produce A and E_2 in amounts even higher than those of hyperplastic stromal cells, it seems reasonable to conclude that the combination of marked stromal hyperplasia and numerous hilus cells in the postmenopausal ovary is unfavourable and may aggravate the above mentioned clinical consequences.

Hilus cell hyperplasia is often associated with clinical signs of virilization (Sternberg 1949). Recently, we studied the steroid production by the ovaries from a markedly virilized postmenopausal woman. Her serum testosterone was approx. 20 nmol/l in repeated determinations (normal range 1-4 nmol/l) and both ovaries showed a marked nodular hilus cell hyperplasia, not only in the hilus region but also in the cortical stroma. When specimens from these ovaries were incubated for 2 h with and without hCG (60 IU/ml) high amounts of A were released into the incubation medium (Fig. 2). There was also a marked responsiveness to hCG in terms of cAMP formation (Fig. 3), this increase in cAMP being much higher than in normal hilus cells (compare Fig. 3 with Fig. 1, paper IV).

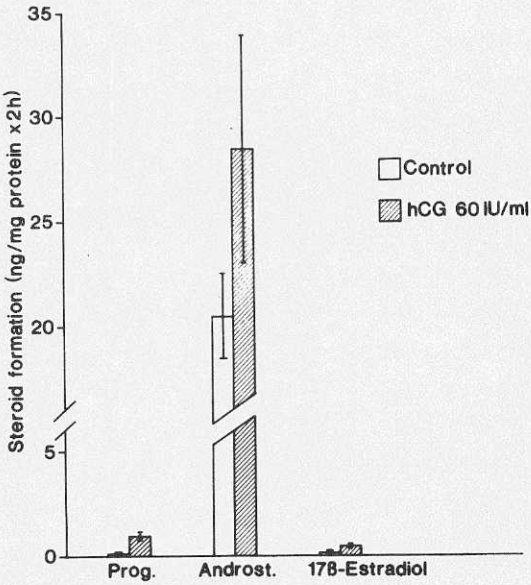

Fig. 2. Release of steroids into the incubation medium from ovarian specimens showing marked hilus cell hyperplasia. Incubation period was 2 h; see text for further details.
Bars indicate means and vertical lines ± SEM.
The stimulatory effects of hCG on the release of P and E_2 are statistically significant ($p < 0.05$).

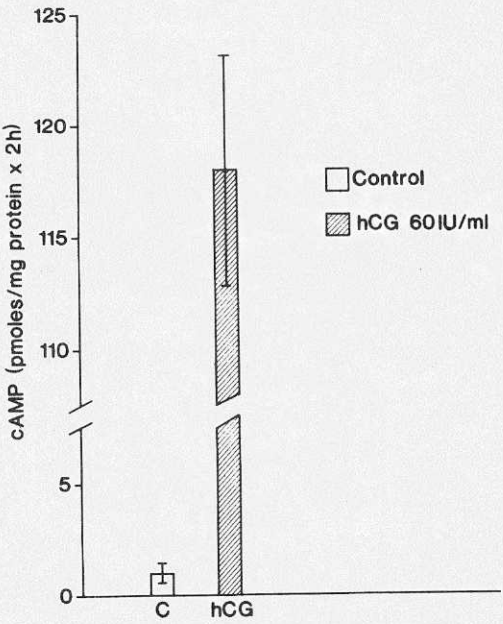

Fig. 3. Tissue formation of cAMP in ovarian specimens with marked hilus cell hyperplasia. Same patient as in Fig. 2.
Bars indicate means and vertical lines ± SEM.
The stimulatory effect of hCG is highly significant ($p < 0.001$).

The similarity between stromal hyperplasia and hilus cell hyperplasia in this respect is obvious and it seems most probable that both disorders are caused by a preserved responsiveness to gonadotropins after the menopause. It remains unclear whether this is due to gonadotropic receptors still present on the cells or to intracellular mechanisms. Future receptor studies of human postmenopausal ovaries may give more insight in the functional variability of the human postmenopausal ovary.

GENERAL SUMMARY

Cells from different compartments of the human ovary were isolated, characterized and utilized for investigations of gonadotropic influence on cAMP formation and steroidogenesis in short-time incubations (30-240 min). In addition, the influence of PG:s on specimens from CL of different ages were studied.

1) Preovulatory follicles: All follicles were carefully examined to assess their degree of development. The two main cell types constituting the follicle, granulosa and theca cells, were mechanically isolated and incubated separately. Both these cell types had the capacity to form all three steroids measured (P, A, E_2). The major steroid formed was A for theca cells and P for granulosa cells, while E_2 was formed in appreciable amounts by both cell types. The results strongly indicate that steroidogenesis is not strictly compartmentalized in the human preovulatory follicle.

Both follicular cell types responded to varying concentrations of hCG with a concentration-dependent increase in cAMP formation. Also the production of P and A by the theca cells, and P by the granulosa cells, was significantly stimulated by hCG *in vitro*. These data demonstrate that both cell types in the preovulatory follicle are acutely responsive to hCG.

2) Corpus luteum: Specimens from CL, dated precisely in relation to the time for ovulation, were incubated and the effects of PGE_2 and $PGF_{2\alpha}$, alone and in combination with hCG, on cAMP and P formation were studied. Human CG stimulated cAMP formation and tended to increase P production in young and old CL. The strongest effects of hCG were noted in CL of the mid-luteal phase, where the formation of both cAMP and P was stimulated. PGE_2 exhibited a stimulatory effect on cAMP formation, both directly and by potentiation of the effect of hCG, albeit only in newly formed CL. $PGF_{2\alpha}$, on the other hand, significantly counteracted the stimulatory effect of hCG on both cAMP and P formation in CL of the mid-luteal phase. In young and old CL $PGF_{2\alpha}$, per se, had no effect on cAMP or P formation, and did not interfere with the effects of hCG. These results indicate that $PGF_{2\alpha}$ has a luteolytic effect in human CL of the mid-luteal phase.

3) Ovarian stroma: Specimens from the cortical stroma of postmenopausal ovaries were examined histologically and signs of stromal hyperplasia were

found in 47% of the ovaries, while in the remaining ovaries the stroma appeared atrophic. Atrophic stroma produced measureable amounts of P, A and E_2 *in vitro*, with A being the predominant steroid formed. Hyperplastic stroma produced 2-fold higher amounts of A and E_2 when compared to atrophic stroma. Human CG elicited a marked stimulation of cAMP formation in hyperplastic stroma but had no effect in atrophic stroma. The results clearly demonstrate that the endocrine function of the postmenopausal ovarian stroma varies with its histological appearance. Hyperplastic stroma exhibits a responsiveness to gonadotropin, has a clear-cut aromatizing capacity, and is steroidogenically more active than atrophic stroma.

4) Hilus cells: Strips from the hilus tissue of postmenopausal ovaries were examined histologically and were found to contain clusters of typical hilus cells. When these specimens were incubated, measureable amounts of P, A and E_2 were produced. A was the major steroid formed, but the formation of E_2 was unexpectedly high. The hilus cells responded to hCG with increased cAMP formation and markedly increased formation of E_2, while P formation was stimulated only slightly. The results demonstrate that the hilus cells of the postmenopausal ovary have a similar, although qualitatively stronger, steroidogenic capacity to the stromal cells and, further, that the hilus cells are capable of responding to gonadotropic stimulation.

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