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Stimulation by hCG of ovarian inactive renin synthesis in rabbit preovulatory theca cells

C. Féral*, Y. Reznik*, S. Le Gall*, J. Mahoudeau*, P. Corvol† and P. Leymarie*

**CNRS URA 609, Laboratoire de Biochimie, Faculté de Médecine, CHU, Côte de Nacre, 14032 Caen Cedex, France; and †INSERM U 36, 17 rue du Fer-à-Moulin, 75005 Paris, France*

Summary. The origin of ovarian renin and its regulation by hCG were investigated in rabbit periovulatory follicles and cultured preovulatory follicular cells. Intracellular content of renin in thecal cells was 8-fold greater than of granulosa cells. *In vivo*, administration of hCG increased intracellular content of renin in thecal but not granulosa cells. Similar results were obtained for cultured follicular cells, from which renin was partly released into the medium. *In vitro*, hCG increased intracellular renin content of thecal but not granulosa cells, without obvious effect on release. Approximately 95% of ovarian renin was inactive, but could be activated by trypsin. Thecal renin was antagonized *in vitro* by renin antiserum, indicating a specific renin activity.

Our study establishes in the rabbit the thecal cell origin of ovarian inactive renin and demonstrates hCG regulation of its synthesis.

Keywords: rabbit; ovary; preovulatory follicle; renin; hCG stimulation

Introduction

Human prorenin, the inactive precursor of renin, is mainly synthesized in the kidney, but the continued secretion of prorenin into the blood after bilateral nephrectomy has implicated production by extrarenal sources (Sealey *et al.*, 1977). Plasma prorenin levels increase about 2-fold during the menstrual cycle at the time of ovulation (Sealey *et al.*, 1985a) and more than 10-fold during pregnancy (Hsueh *et al.*, 1982; Sealey *et al.*, 1982). Although placental choriodecidual cells are known to synthesize prorenin (Symonds *et al.*, 1968; Poisner *et al.*, 1982; Acker *et al.*, 1982; Shaw *et al.*, 1989), the pregnancy-related rise in plasma prorenin cannot be due to placental secretion since the prorenin level only decreases several days after parturition, much later than the drop in placental gonadotrophin (hCG) level (Sealey *et al.*, 1985b). Moreover, no prorenin rise was observed in pregnant women with primary ovarian failure who conceived with donor eggs (Sealey *et al.*, 1986; Derkx *et al.*, 1987). An ovarian origin of plasma extrarenal prorenin has been demonstrated in the baboon (Sealey *et al.*, 1986). Culture of human follicular cells isolated from premenopausal women showed an in-vitro secretion of prorenin by theca cells but not by granulosa cells (Do *et al.*, 1988).

Measurements of renin mRNA in rat ovarian extracts have shown that its production could be regulated by FSH (Kim *et al.*, 1987). However, clinical data suggest that prorenin secretion may be regulated by LH/hCG since the plasma prorenin increase observed during the human menstrual cycle occurs 6 h after the LH surge (Sealey *et al.*, 1987) and the rise during pregnancy is in parallel with the rise in hCG (Sealey *et al.*, 1985b).

As a useful system to explore the mechanisms that control ovarian renin synthesis we chose the female rabbit, a reflex ovulator whose ovaries offer a constant supply of preovulatory follicles; moreover, the thecal tissue can be efficiently separated from the granulosa.

The aim of the present study was to investigate a possible regulatory role of hCG in synthesis of renin in the rabbit ovarian follicle.

Materials and Methods

Animals and treatment

Virgin HY white rabbits, 12–15 weeks old, 2·5–3 kg body weight (Elevage Vivien, Fontenay-le-Marmion, France) were used for these studies. They were housed individually for about 2 weeks on a 14-h light (06:00–20:00 h) and 10-h dark schedule with standard rabbit food and water available *ad libitum*.

The development of 10–15 preovulatory follicles per ovary was induced in immature rabbits by s.c. injection of 100 i.u. PMSG (Chrono-Gest: Intervet International, Boxmeer, Netherlands). The animals were killed by cervical dislocation 65 h after injection. Periovulatory follicles were obtained in a second group of immature females by administration of 100 i.u. PMSG followed 65 h later by i.m. injection of an ovulatory dose (100 i.u.) of hCG (Pregnyl, Organon, Sérifontaine, France). The rabbits were killed 3 h after hCG injection. The ovaries were excised and placed in iced culture medium. Follicles were dissected out; those containing clotted blood were discarded and the rest were cleared of interstitial tissue stereomicroscopically.

Cell preparation

Isolation of granulosa cells. The culture medium was Medium 199 with Earle's salts supplemented with 2·5% FCS (Seromed, Lyon, France), buffered with 20 mM-Hepes and including 50 i.u. penicillin/50 μg streptomycin/ml. The medium was sterilized by filtration through a 0·22 μm type GS filter (Millipore, Molsheim, France). Dispersed cells were obtained by a procedure based on that described by Campbell (1979): follicles were placed in serum-free Medium 199 containing 0·1% BSA and 6·8 mM-EGTA (Sigma Chimie, La Verpillière, France), punctured with a fine insect pin to allow entry of EGTA, and maintained at room temperature for 15 min. The released granulosa cells and the follicles were then centrifuged together at 300 *g* for 10 min at room temperature and resuspended in Medium 199-BSA containing 0·5 M-sucrose and 1·8 mM-EGTA. They were incubated for an additional 5 min and centrifuged at 300 *g* for 10 min. After removal of the hypertonic sucrose medium, the punctured follicles were placed in fresh Medium 199-BSA and cut in half. Granulosa cells sticking to the inner surface of the follicle were gently scraped away from the basement membrane with a microspatula until no remaining cells were observed under the dissecting microscope. All granulosa cells (puncture-released cells + scraped cells) were pooled and centrifuged at 300 *g* for 10 min and subjected to enzymic dissociation: the clusters of granulosa cells were suspended in 10 ml serum-free Medium 199-BSA containing 0·05% collagenase–dispase (Boehringer-Mannheim, Meylan, France), 0·05% hyaluronidase (Type II, Sigma), 5 mg trypsin inhibitor (Type II O, Sigma), ~1 mg DNase (Type I, Sigma) and incubated at 37°C in a shaking water bath for 30–45 min. Dissociation of the cells was assisted by repeated pipetting of the suspension with a Pasteur pipette.

At the end of the incubation, the cell suspension was diluted 1:1 with Medium 199-BSA and centrifuged for 10 min at 300 *g*. The dispersed cells were washed twice and resuspended to a concentration of approximately 2×10^5 cells per ml in Medium 199 supplemented with FCS. Cells were plated in a total volume of 4 ml medium in 25 cm^2 plastic tissue culture flasks. Each flask contained granulosa cells equivalent to 10 follicles.

Preparation of thecal pieces. Efficient removal of granulosa cells from the thecal wall was confirmed by surface scanning electron microscopy (Boyde & Wood, 1969). Representative samples of follicular wall taken from several replicate experiments before and after scraping of granulosa cells were fixed in glutaraldehyde and osmium tetroxide, critical-point dried, coated with gold and observed in a scanning electron microscope (Cambridge Scanning, Siemens, St Denis, France). A possible remaining contamination by granulosa cells was eliminated during further preparation. Thecal layers were brought into suspension by a modification of the procedure reported by Tonetta *et al.* (1987): briefly, follicle walls were minced with iris scissors and pieces were incubated with stirring in PBS containing 0·1% collagenase (type II, Sigma) and 0·1% hyaluronidase (type II, Sigma) for 30 min at 37°C. After addition of 10 ml Medium 199-BSA, they were centrifuged (600 *g*) for 30 sec.

The supernatant consisting of the remaining granulosa cells was discarded. The small thecal pieces (0·02 mm^3) cleared of granulosa cells were transferred to 25 cm^2 culture flasks in 4 ml Medium 199-FCS. Each flask contained thecal tissue equivalent to 10 follicles. To examine the contamination of steroidogenic theca cells by other cell types, the thecal pieces were resuspended in 0·1% protease (type XIV, Sigma) and 0·2% hyaluronidase (type II, Sigma) in PBS and incubated for 45 min at 37°C. Medium 199-BSA was added. Cells were then centrifuged (300 *g*, 10 min) and washed twice.

The final cell suspension consisted of theca cells but also contained fibroblast-like non-steroidogenic cells. Identification of theca cells was accomplished by staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity using the procedure described by Browning *et al.* (1981). Approximately 70% of the cells in the suspension stained lightly for 3β-HSD activity and were theca cells. Because of the heterogeneity of the cell suspension, the rates of steroid or renin secretions were normalized per follicle.

Cell culture

Granulosa cells and thecal pieces were cultured in Medium 199-FCS in the presence and absence of hCG (0·1 i.u./ml) at 37°C, under 5% CO_2 and 95% air. Medium was removed at 24 h. One part of the spent medium was stored at −20°C for determination of steroids and another part with 5×10^{-3} M-EDTA/10^{-2} M-NaOH at −80°C for determination of renin content. The cells were harvested mechanically by scraping into PBS and the cell pellet was used for intracellular renin assay.

Steroid assay

The production of progesterone and 4-androstenedione in the culture medium was determined by RIA. Before extraction, a tracer (~1000 c.p.m. progesterone or ~1000 c.p.m. 4-androstenedione) was added for recovery estimation. Steroids were extracted twice from culture medium with 5 volumes of distilled ether. Dried extracts were dissolved in 1 ml isooctane and chromatographed on 0·5 × 5 cm celite microcolumns (Chromatolithe A, Bio-Merieux, Charbonnières-les-Bains, France) using the solvent system isooctane for progesterone and ethylacetate/isooctane (6/94) for androstenedione. The fractions containing progesterone and 4-androstenedione were evaporated in a speed vacuum concentrator and assayed by RIA as previously outlined (Ursely & Leymarie, 1979). The inter- and intra-assay coefficients of variation (CVs) were 5% and 9% respectively for both steroids and the sensitivity was 6 pg/tube for progesterone and 3 pg/tube for 4-androstenedione.

Renin assay

Preparation of cell lysate. Cells were rinsed and scraped off the flasks. For active renin measurement, they were resuspended in 0·5 ml phosphate buffer containing protease inhibitors to avoid in-vitro activation of inactive renin. As described by Murakami & Inagami (1975) the following protease inhibitors were dissolved in 0·1 M-phosphate buffer, pH 7·5: 5 mM-sodium tetrathionate, 50 μM-diisopropylfluorophosphate (Fluka, Mulhouse, France), 10 mM-*N*-ethylmaleimide (Aldrich, Strasbourg, France), 10 mM-benzamidine, 2 mM-ethylene-diaminetetraacetate disodium salt (Sigma Chimie, La Verpillière, France) and 2 mM-phenylmethylsulphonyl fluoride (Merck, Nogent-sur-Marne, France). For total (inactive + active) renin measurement, cells were resuspended in 0·5 ml PBS (pH 6) without protease inhibitors. Cells were stored at −80°C and they were sonicated 3 times for 30 sec at 4°C. Aliquants were stored at −80°C until renin assay.

Assay of enzyme activity. The renin activity in the extracts, culture medium or plasma was assayed by determining the angiotensin I-generating activity. Lysates, cell culture medium or plasma (25 μl) were incubated for 1 h at 37°C, pH 6·5, with 250 μl plasma from anephric rats as substrate. Generated angiotensin I was determined by RIA (Menard & Catt, 1972).

Total renin (active + inactive) was measured after an activation by trypsin treatment using final concentrations of 0·001–5 mg trypsin/ml (type XI, Sigma). Cell extract, cell culture medium or plasma (25 μl) were incubated for 1 h at 4°C with 1 μg trypsin in 25 μl 0·1 M-phosphate buffer, pH 7·5. The reaction was stopped by adding 100 μg soybean trypsin inhibitor (Sigma) in 100 μl 0·1 M-phosphate buffer/10^{-3} M-EDTA, pH 6·5. In a few experiments, active renin was assayed from samples stored with protease inhibitors to determine active/inactive renin ratios. The proportion of inactive renin was deduced from the difference between total and active renin measurements. Renin concentrations were expressed as pg angiotensin I/h/follicle. The intra- and inter-assay CVs for RIA were 8% and 12% respectively. Assay sensitivity averaged 7 pg angiotensin I/h/tube. All samples of each experiment were assayed together.

Characterization of ovarian renin

Optimum pH. Cell extracts stored with protease inhibitors (active renin) and trypsin-treated cell extracts (activated renin) were incubated with plasma, from anephric rats, in phosphate buffer at various pH values (5–8). The renin activity was then determined as described above.

Determination of enzyme kinetics. Thecal renin was incubated at pH 6·5 with plasma of nephrectomized rats as substrate: 25 μl of thecal cell extracts were incubated with increasing concentrations of plasma (angiotensinogen concentration 640 to 1920 ng angiotensin I equivalent/ml) at 37°C and pH 6·5 for 1 to 4 h. The K_m value was determined by Lineweaver–Burke plot analysis.

The plasma angiotensinogen concentration was determined by addition of excess exogenous renin to the nephrectomized rat plasma at pH 6·5 and incubation at 37°C to substrate exhaustion (1 h).

Renin inhibition by renin antiserum. To examine whether trypsin treatment activated inactive renin rather than a non-renin enzyme, trypsin-treated cell lysates (after addition of soybean trypsin inhibitor) were incubated overnight at 4°C with a mouse renin antiserum, at various dilutions ranging from 10^{-2} to 10^{-5}. The renin activity was then determined.

Statistical analysis

Results, given as the mean ± s.e.m., were analysed by Student's *t* tests.

Results

Characterization of cultured granulosa and theca cells

Separation of granulosa cells and thecal wall was verified by scanning electron microscopy. Examination of 4 follicles revealed that contamination of thecal tissue by granulosa cells was already negligible after the scraping of granulosa cells from the thecal wall (<1% of the mean granulosa cell count per follicle). The few remaining granulosa cells were eliminated during the enzyme treatment of thecal pieces.

Efficient separation of granulosa and theca was confirmed by measurements of 4-androstenedione and progesterone in the culture medium. The granulosa cells produced higher levels of progesterone and much lower levels of androgen compared to theca cells (Fig. 1). The progesterone/4-androstenedione ratio was 1600 for the granulosa cell medium compared to 8 for the theca cell medium. The addition of 0·1 i.u. hCG/ml to the culture medium led to an 11-fold elevation of progesterone in granulosa cell medium, and to a 10-fold increase of 4-androstenedione in theca cell medium compared to control cultures. This is consistent with the known relative pattern of progesterone/androgen production in ovarian granulosa cells and thecal tissue in the rabbit (YoungLai, 1972; Erickson *et al.*, 1974; Erickson & Ryan, 1976).

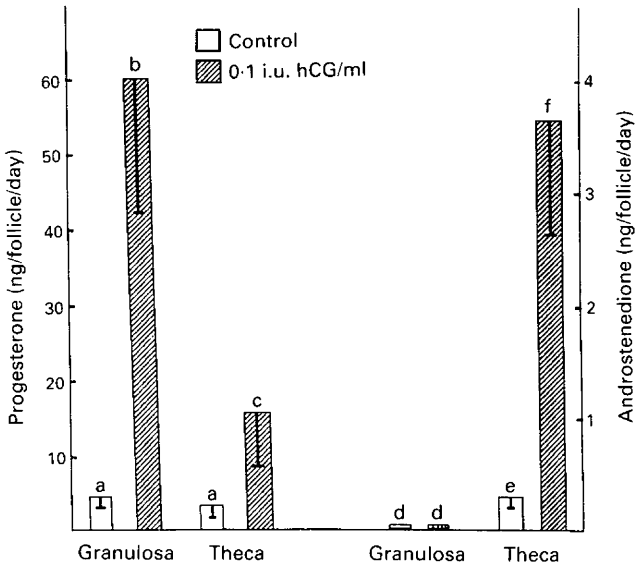

Fig. 1. Characterization of granulosa cells *versus* thecal pieces by their in-vitro steroid secretion. Cells were grown for 24 h. Values are the mean ± s.e.m. of duplicate determinations in 4 experiments. a *vs* b and c, b *vs* c, d *vs* e, e *vs* c, $P < 0{\cdot}002$.

Effect of hCG injection on endogenous renin content of follicular cells

Total renin was measured in preovulatory follicles isolated from ovaries of immature PMSG-treated rabbits (Fig. 2). Significantly higher amounts of renin were found in the theca cell extracts compared to that in granulosa cells ($P < 0{\cdot}001$). The ratio of theca/granulosa renin content was about 8. In the periovulatory follicles induced by hCG injection in immature PMSG-treated rabbits (Fig. 2), the amount of renin in theca cells was about 2·5-fold greater than that in PMSG-treated controls ($P < 0{\cdot}01$). In contrast hCG induced no significant change in renin content of granulosa cells.

Total renin measurements in plasma of 3 rabbits before and 3 h after hCG administration showed a significant rise in plasma total renin ($P < 0{\cdot}01$). No change in plasma total renin of the same rabbits was apparent before and after PMSG injection.

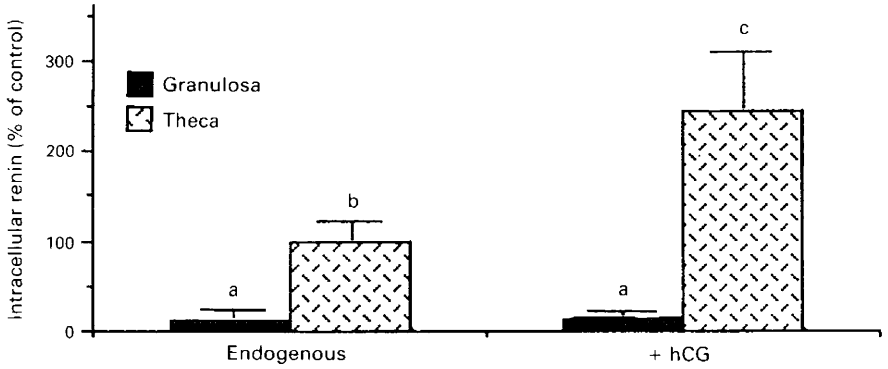

Fig. 2. Endogenous total renin content of ovarian follicular cells from immature PMSG-treated control rabbits and 3 h after i.m. injection of 100 i.u. hCG. Results are expressed as the mean ± s.e.m. of triplicate determinations in 14 rabbits. a *vs* b, $P < 0{\cdot}001$; b *vs* c, $P < 0{\cdot}01$.

Characterization of rabbit ovarian renin

Less than 5% of the total renin content in the theca cells was in an active form, as shown by the low or undetectable renin activity measured in cell extracts stored in protease inhibitors containing buffer. Hence, rabbit ovarian renin was more than 95% inactive. Optimal pH values of the ovarian active and activated renin for hydrolysing the rat angiotensinogen were identical (pH 6·5). The Michaelis–Menten kinetic constant (K_m) of thecal renin was 1·7 μM. This was similar to those of rabbit renal and uterine renin, i.e. 1·7 and 1·8 μM respectively (Dzau *et al.*, 1987). Ovarian active renin obtained by trypsin treatment of theca cell lysates was completely inactivated by an antiserum against mouse submaxillary gland renin with a 50% inhibition of the enzyme activity occurring at 7×10^{-3} dilution of the antiserum, demonstrating that the observed renin activity was due to the specific action of renin.

Effect of hCG on renin synthesis by cultured follicular cells

Total renin was measured in 24-h cultured theca and granulosa cells isolated from immature PMSG-treated rabbits and compared with the results of the in-vivo study (Fig. 3). After 24 h culture, high levels of renin were found in the theca cell extracts, and extremely low quantities in the granulosa cell extracts ($P < 0{\cdot}01$). Theca/granulosa renin ratio was about 10. Moreover, intracellular renin in theca cells cultured for 24 h (control) did not differ from intracellular renin in non-cultured cells (endogenous).

Incubation for 24 h with 0·1 i.u. hCG/ml increased thecal intracellular renin by 415 ± 86% compared to that in control culture containing no hormone ($P < 0{\cdot}01$) (Fig. 3). Absolute intracellular renin content ranged from 0·2 to 7 ng angiotensin/h/follicle in control cells and from 1·5 to 17 in stimulated cells. *In vitro*, hCG induced a net increase of renin in theca cells but no significant change in renin content of granulosa cells (Fig. 3). Between 20 and 40% of intracellular renin was released into the culture medium of theca cells, whereas no renin was detectable in culture medium of granulosa cells. The addition of hCG induced no increase in renin activity in culture medium of theca cells compared with the controls.

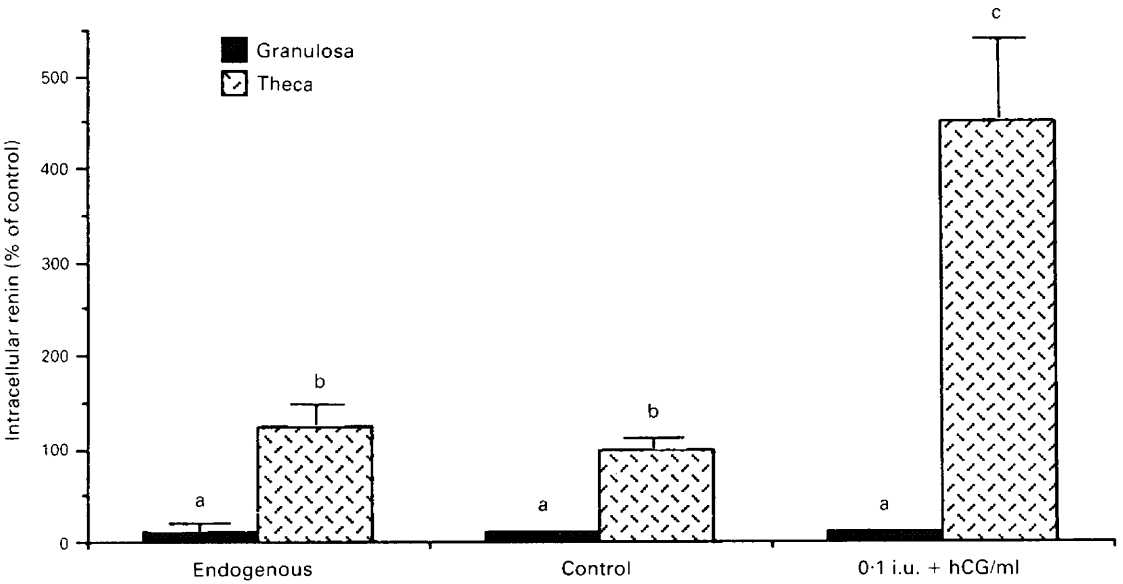

Fig. 3. Intracellular total renin in cultured granulosa and theca cells from immature PMSG-treated rabbits. Cells were grown for 24 h. The endogenous renin in non-cultured cells is also given for reference. Data are the mean ± s.e.m. from triplicate determinations in 4 experiments. a *vs* b and b *vs* c, $P < 0{\cdot}01$.

Discussion

The present study provides the first demonstration that rabbit theca cells contain renin and that hCG stimulates renin synthesis both *in vivo* and in primary culture.

High levels of renin were found in preovulatory theca cells compared to low amounts in granulosa cells. These findings in the rabbit are in agreement with data reported for the human (Do *et al.*, 1988) in which prorenin is secreted *in vitro* by theca cells but not by granulosa cells, for the rat (Howard *et al.*, 1988) in which renin remained in the residual ovarian tissue after the granulosa cells had been expressed and for the cow (Schultze *et al.*, 1989) in which high levels of renin were found in theca cells. The appreciable amounts of prorenin found in granulosa cells from women undergoing in-vitro fertilization (Glorioso *et al.*, 1987) might have been due to the uptake by granulosa cells of the renin thecal secretion.

As in human (Sealey *et al.*, 1986; Do *et al.* (1988), the renin produced by rabbit theca cells was about 95% inactive but could be activated by trypsin. Howard *et al.* (1988) detected high levels of active *versus* inactive renin in the rat ovary; they suggested that methodological differences might explain such a discrepancy with previous studies. The rabbit activated renin was fully antagonized *in vitro* by an antirenin antibody, indicating that the renin activity was due to the specific action of ovarian renin rather than the non-specific action of another protease. Treatment of rabbits with hCG induced a significant 2·5-fold increase of renin in theca cells while the small amount of renin in granulosa cells was not enhanced. At the same time, renin level increased in plasma, suggesting that renin might be synthesized or secreted in response to hCG injection.

In rabbit theca cells cultured for 24 h, the intracellular renin level did not differ from the endogenous content of non-cultured cells, whereas hCG induced a 4-fold increase. Such an increase obtained *in vitro* provides an unambiguous demonstration of a net synthesis of renin by theca cells.

Thecal synthesis of renin has not yet been proved; only an in-vitro secretion of prorenin by theca cells has been demonstrated in women (Do *et al.*, 1988). Our observations in the rabbit

indicate that about 70% of renin was confined inside the cell while only a small portion was released into the culture medium. In the present experiments, hCG does not seem to stimulate renin release.

The present demonstration of an in-vitro stimulation of rabbit thecal renin synthesis by hCG is consistent with previous clinical observations of an increase in human plasma prorenin at the time of the ovulatory surge of LH (Sealey *et al.*, 1987) and throughout pregnancy (Sealey *et al.*, 1985b). On the other hand, Kim *et al.* (1987) reported that ovine FSH induced a 3-fold increase in rat ovarian renin mRNA *in vivo* and suggested that expression of the ovarian renin gene may be regulated by FSH (no data were available concerning the effect of LH). However, it is questionable whether theca cells are capable of responding to FSH by an increase in renin production since, as far as we know, theca cells bear no specific FSH receptors (Zeleznik *et al.*, 1981). Hence, the FSH stimulation of rat ovarian renin mRNA (Kim *et al.*, 1987), if not due to a trace amount of contaminating LH in the ovine FSH preparation, would have to reflect an indirect effect of FSH on theca cells via granulosa/theca cell interactions.

In the rat and human testis, immunohistochemical studies (Parmentier *et al.*, 1983; Naruse *et al.*, 1985) and enzyme activity assays (Pandey *et al.*, 1984) have revealed that Leydig cells contain renin. Leydig and theca cells share the same embryological origin and analogous endocrine functions; hCG stimulates renin synthesis in theca cell just as it induces renin production in mouse Leydig tumour cells *in vitro* (Pandey *et al.*, 1985).

The ovary contains all the components of the renin angiotensin system: prorenin and its mRNA (Kim *et al.*, 1987), angiotensinogen and its mRNA (Ohkubo *et al.*, 1986), angiotensin converting enzyme, angiotensin II and angiotensin II-receptor binding sites (Speth & Husain, 1988; Husain *et al.*, 1987). The demonstration of regulation of renin synthesis by hCG in rabbit theca cells supports the concept of a functional ovarian renin angiotensin system.

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