BIOLOGY OF REPRODUCTION 25, 421–431 (1981)

Granulosa Cell Differentiation in vitro: Effect of Insulin on Growth and Functional Integrity[1]

JEFFREY V. MAY and DAVID W. SCHOMBERG[2]

Department of Obstetrics and Gynecology and Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

Insulin is a requisite for the FSH-mediated induction of LH/hCG receptors in porcine granulosa cell monolayers maintained in serum-containing medium (May et al., 1980). In this report we describe further the role of insulin on this process and on other parameters of cell monolayer performance, e.g., plating efficiency, cell growth, basal and gonadotropin-stimulated progesterone production, and aromatase activity.

Insulin did not affect the plating efficiency (>90%) of freshly harvested, viable granulosa cells. During the first 2 days of culture, monolayer cell content increased slightly under insulin-free conditions. Insulin did not alter this slow rate of cell division. After the first 2 days, however, cell and protein content of monolayers deprived of insulin decreased significantly (60%, $P<0.001$) relative to insulin-treated monolayers in which cell and protein content were either maintained or increased relative to the initial innoculum.

Basal progesterone secretion declined with time in culture but was maintained or slightly increased under the influence of insulin. Both FSH- and hCG-stimulated progesterone production were significantly enhanced ($P<0.001$) by insulin after the first 2 days of culture. FSH-stimulated progesterone secretion was dose-dependent with respect to insulin as was FSH-mediated LH/hCG receptor induction.

The immature granulosa cells used in these studies assumed a fibroblastic morphology in the absence of added hormones. Insulin induced an epithelioid morphology in vitro, characteristic of more mature cells. LH and FSH alone were incapable of altering the fibroblastic morphology and did not affect the change brought about by insulin. Thus, gross morphological maturation of granulosa cells in vitro correlated with insulin-mediated biochemical differentiation, e. g., LH/hCG receptor induction and steroidogenic capacity.

Although insulin enhanced several markers of cell differentiation, it did not ameliorate the loss of aromatase activity during culture, a characteristic of this model system. During the first 2 days of culture and in the presence of testosterone, granulosa cell monolayers secreted significant amounts of estrogen. However, this capability declined rapidly with time in culture. Neither insulin alone nor insulin combined with FSH acutely stimulated aromatase activity, nor did these treatments protect against the loss of aromatase activity.

Highly purified porcine relaxin and commercially available multiplication-stimulating activity (MSA), compounds possessing insulin-like structure and activity, respectively, were incapable of replacing insulin as a requisite for FSH-mediated LH/hCG receptor induction or gonadotropin-stimulated progesterone secretion.

Our results indicate that insulin is critical for the maintenance of several functional properties of porcine granulosa cell monolayers, and thus should be considered for routine use in studies of the regulation of ovarian function in vitro.

INTRODUCTION

Isolated cell and tissue culture systems are being used increasingly as a direct means of studying ovarian regulation because selected markers of cellular differentiation can now be maintained in vitro. Granulosa cells from numerous species have been used to study such aspects as steroid biosynthesis, gonadotropin receptor regulation, intercellular communication, and cell growth. Many of these studies have utilized nutrient medium supplemented with serum, the role of which is to provide specific nutrients, growth factors, and hormones which facilitate cell attachment, maintenance, and growth (Ham and McKeehan, 1979; Barnes and Sato, 1980). This hypothesis has largely been substantiated by Sato and his

Accepted May 6, 1981.
Received February 23, 1981.
[1] This work was supported in part by grant no. HD 11827 from the NICHD, USPHS.
[2] Reprint requests: Dr. David W. Schomberg, Box 3323, Duke University Medical Center, Durham, NC 27710.

colleagues who have reported that for several cell lines, a defined set of hormones and growth factors can replace serum (Hayashi et al., 1978; Mather and Sato, 1979). Although individual requirements varied among these lines, insulin was found to be a growth requisite for virtually every cell type studied.

The decision to utilize serum-containing vs serum-free medium for the study of granulosa cell function in vitro may depend, in part, upon the endocrine environment of the cells in vivo. Erickson et al. (1979) demonstrated follicle stimulating hormone (FSH)-mediated induction of luteinizing hormone (LH) receptors in granulosa cells from immature, diethylstilbestrol (DES)-primed hypophysectomized rats during 2-day cultures maintained in serum-free medium devoid of insulin. Serum was shown to be inhibitory in this system which explained why earlier attempts to induce LH/hCG receptor in rat granulosa cell monolayers failed (Nimrod et al., 1977). Orly et al. (1980), using the same rat model, reported that serum also inhibits FSH-stimulated steroidogenesis. Studies in our laboratory utilizing cells from *intact,* immature, DES-primed rats, have confirmed that serum is detrimental to LH/hCG receptor induction and that induction under serum-free conditions requires insulin during moderate-term culture (Mondschein and Schomberg, 1981).

Serum is not uniformly inhibitory to LH/hCG receptor induction in all granulosa cells, however. We have reported that FSH can induce LH/hCG receptors in porcine granulosa cell monolayers maintained in serum-containing medium and that insulin is a requisite (May et al., 1980). Serum was utilized in these studies to facilitate cell growth and attachment since the plating efficiency of porcine cells in serum-free medium is extremely poor (Channing et al., 1976). Apart from the difference in serum sensitivity, our studies indicated that granulosa cells from both the pig and estrogen-primed, intact, immature rat required insulin for LH/hCG receptor induction during moderate-term, primary, monolayer culture.

In view of the requirement for insulin with respect to receptor induction, we undertook studies to determine its effects on other aspects of granulosa cell development in the presence of serum. Our results indicate that insulin has profound effects on monolayer growth, maintenance, and morphology, as well as on differentiated cellular functions such as receptor induction and basal and gonadotropin-stimulated steroidogenesis.

MATERIALS AND METHODS

Porcine ovaries from adult, cycling animals were obtained from a local slaughterhouse and placed into cold phosphate-buffered saline (PBS; pH 7.8) supplemented with penicillin, streptomycin, mycostatin, and fungizone [20U/ml, 20μg/ml, 20 U/ml, and 0.5 μg/ml, respectively, Grand Island Biological Co. (Gibco), Grand Island, NY]. Intact hemifollicle linings from 1–3 mm follicles were removed, pooled, and the granulosa cells isolated by mechanical agitation as previously described (Haney and Schomberg, 1978) with slight modification in that individual follicles were not abraded against the wall of the culture tube. The isolated hemilinings were agitated with a glass rod, using a rapid up-and-down motion for 2 min. Following this, they were allowed to settle and the cell-containing medium was removed with a sterile pasteur pipet and filtered through a 25 mm Swinnex filter (Millipore Corp., Bedford, MA) containing 177 μm nylon mesh screen. Fresh medium was added to the follicles and the procedure repeated except that the entire content of the tube was filtered as above. Cell viability after this procedure, as determined by trypan blue exclusion, was 53.2 ± 2.2% (mean ± SEM). Cell numbers following initial harvest or after culture were obtained by direct counting using a hemacytometer; cultured cells were detached from the multiwells (Falcon Plastics, Los Angeles, CA) by a 5 min treatment with 0.2 ml of 0.25% trypsin in 5 mM EDTA. The trypsin was subsequently neutralized by the addition of serum-containing medium.

Granulosa cells were cultured as monolayers in Hams F-12 nutrient medium (Gibco) supplemented with 10% (v/v) fetal calf serum (Gibco) as previously described (May et al., 1980). Media changes were performed as indicated in the text and media progesterone and/or estrogen content were determined by radioimmunoassay (RIA) as described by May et al. (1980) and Aksel et al. (1976), respectively.

Porcine insulin (ID G04-94-193, 25 mIU = 1 μg) was a gift from the Eli Lilly Co. (Indianapolis, IN). Highly purified human FSH (LER-1577) was provided by Dr. Leo E. Reichert, Jr., Albany Medical College. Human chorionic gonadotropin (hCG, mean activity 16,533 IU/mg, based upon rat Leydig cell testosterone production, radioimmunoassay and radioreceptorassay relative to hCG-CR119, 15,000 IU/mg) was provided by Dr. David Puett, Vanderbilt University. Highly purified porcine relaxin (lot no. 18) was supplied by Dr. O. David Sherwood, the University of Illinois. Multiplication-stimulating activity (MSA; lot 1084–70) was obtained from Collaborative Research, Inc. (Waltham, MA). Solutions of insulin, hCG, FSH, relaxin, and MSA were freshly made for each experiment. Each was dissolved in sterile PBS and stored at 4°C in sterile plastic culture tubes. Steroids were obtained from Steraloids, Inc. (Wilton, NH) and dissolved in absolute ethanol.

LH/hCG receptor content of granulosa cells and monolayers was estimated by the specific binding of [^{125}I] iodo-hCG (routine sp act 33.5 ± 2.0 μCi/μg, mean ± SEM) as described by May et al. (1980), using hormone labeled according to Stouffer et al. (1976).

RESULTS

Effect of Insulin on Granulosa Cell Growth and Maintenance

We previously reported that an important marker of porcine granulosa cell development, i.e., FSH-mediated LH/hCG receptor induction, could be achieved in vitro in serum-containing medium supplemented with insulin (May et al., 1980). Although not studied in detail, an overall beneficial effect of insulin upon cell growth and maintenance was also noted in this study. To investigate this aspect in greater detail, porcine granulosa cells from 1–3 mm follicles were cultured as monolayers in medium alone or medium supplemented with insulin. Cell number and protein content were determined after various times in culture.

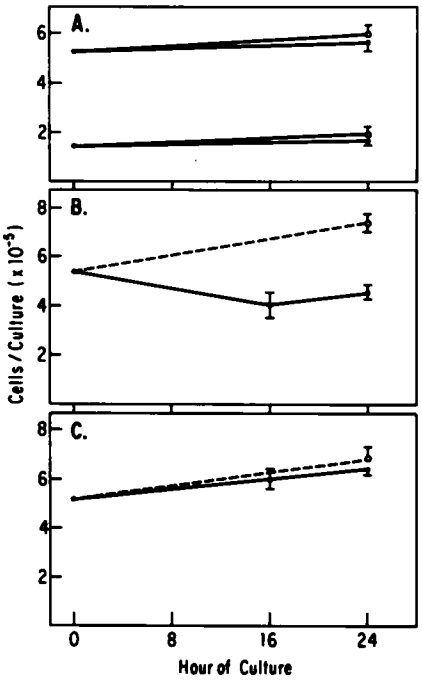

FIG. 1. Effect of insulin on monolayer cell number during the early stages of culture. Granulosa cells from 1–3 mm follicles were used.

A) Cells (5×10^5 and 1.5×10^5) were cultured as monolayers in Ham's F12 nutrient medium + 10% fetal calf serum with (open circles) or without (closed circles) insulin (25 mIU/ml) for 24 h. Monolayer cell number was determined as described in the text.

B,C) In replicate experiments, granulosa cells (5×10^5) were cultured with insulin for 24 h without interruption (dashed line). Other monolayers were washed vigorously (B) or gently (C) to remove dead, nonattached, or loosely attached cells. The monolayer cell content of half of these 16 h cultures was determined (solid line) while the remaining half received fresh insulin-containing medium and was reincubated for an additional 8 h. At the end of this period, monolayer cell number was determined. All points represent the mean ± SEM of triplicate cultures.

After 24 or 48 h of culture (Figs. 1, 3, respectively) an insulin-independent increase in monolayer cell number relative to the initial innoculum was noted. While this increase was not always statistically significant, a definite trend was established. During these early stages of culture, the monolayer cell population reflects both cell attachment and mitotic activity. Conceptually, an increase in monolayer cell number could be derived from varying quantitative contributions of these two parameters. To characterize the contribution of these aspects to monolayer population dynamics, granulosa cells (5×10^5) were maintained for 16 or 24 h in insulin-supplemented medium. The 16 h cultures were washed to remove nonattached and dead cells; attached cells were then counted in half of these cultures following enzymatic detachment. The remaining cultures were reincubated in fresh medium for an additional 8 h at which time cell numbers were again determined. Comparisons of cell numbers from these 16 and 24 h washed cultures with those from 24 h uninterrupted cultures are shown in Fig. 1b,c. In the 24 h uninterrupted cultures (open circles, dotted line), total cell number increased 1.3–1.4-fold, reflecting both cell attachment and mitotic activity. Monolayers washed vigorously (Fig. 1b) or gently (Fig. 1c) at 16 h and reincubated for an additional 8 h exhibited a small increase in cell number during this period (solid circles, solid lines). Since nonattached cells had been removed at 16 h, the increased cell count at 24 h can only represent mitotic activity. Although the increase in monolayer cell number during this subsequent 8 h period was not statistically significant relative to 16 h, the slope of the line representing this increase (6.8×10^3 cells/hour) very nearly parallels that representing the increase in cell number during 24 h of uninterrupted culture (8.2×10^3 cells/hour), suggesting the growth rates were similar if not identical. These indirect analyses indicate that granulosa cells from small porcine follicles exhibit a slow growth rate and plate at high efficiency (>90%). The 20–25% increase in the cell number per day would suggest an approximate 4–5 day doubling time for the monolayer population.

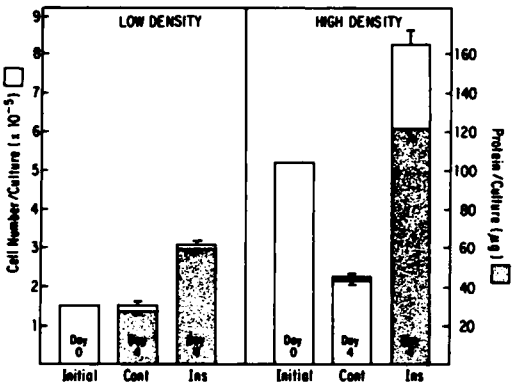

FIG. 2. Monolayer growth response to insulin: Effect of plating density. Granulosa cells of low (1.5 × 10^5) and high (5 × 10^5) density plating were maintained for 4 days in medium with or without insulin (25 mIU/ml). Monolayer cell number and protein content were then determined. Bars represent the mean ± SEM of triplicate cultures.

Although insulin did not affect attachment and growth during the early stages of culture, it dramatically influenced monolayer growth and maintenance during later stages (Fig. 2). Granulosa cells at two different innoculum densities were cultured for 4 days with or without insulin. At the lower innoculum density (1.5 × 10^5 cells), cell number doubled in 4 days in the presence of insulin but remained constant in control cultures. At the higher innoculum density (5 × 10^5 cells), i. e., that routinely used for studies of LH/hCG receptor regulation in this laboratory (May et al., 1980), cell number again increased in the presence of insulin. In control cultures, however, the cell number decreased dramatically to a level 3.7-fold below that of insulin-treated cultures. In both insulin-treated and control cultures, the protein content generally reflected cell number, and extensive cellular hypertrophy was not observed.

To test further the importance of insulin in vitro, cell growth and maintenance of granulosa cells were monitored temporally during 6 day cultures. During the first 2 days, insulin had little, if any, effect on the monolayer cell population (Fig. 3). After 2 days, however, the cell number of insulin-free cultures declined markedly relative to insulin-treated cultures (58%, $P<0.001$). Thus, insulin appears to benefit granulosa cells during a critical period of cell survival in vitro. A maintenance effect of insulin with regard to monolayer cell and protein content was observed in this study rather than the insulin-mediated increase in cell number and protein content normally observed (Fig. 2). The reason for this difference in response is not known but may reflect a spectrum in growth potential by cells from given harvests due to varying proportions of putative developing vs atretic follicles.

Effect of Insulin on Granulosa Cell Morphology

Immature granulosa cells from small antral follicles assume a fibroblastic appearance in vitro, whereas granulosa cells from mature, preovulatory follicles assume an epithelioid morphology and exhibit other characteristics of steroid-secreting cells (Channing, 1970). Representative phase-contrast photomicrographs of immature granulosa cells maintained for 3 days in serum-containing medium supplemented with combinations of insulin, FSH, and hCG are presented in Fig. 4.

Granulosa cells assumed a fibroblastic morphology in serum-containing medium (Fig. 4A) which was not altered by FSH (30 ng/ml) or hCG (100 ng/ml) (Fig. 4C, E, respectively). Insulin, however, dramatically altered cellular appearance, inducing an epithelioid morphology (Fig. 4B) characteristic of more highly differentiated cells. The insulin effect was not

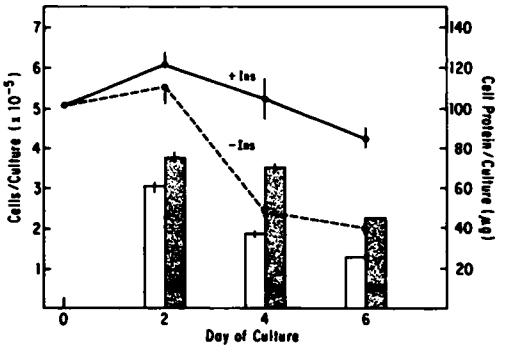

FIG. 3. Effect of insulin on monolayer cell number and protein content as a function of time in culture. Granulosa cells were maintained for 2–6 days in medium with (solid line and shaded bars) or without (dashed line and open bars) insulin (25 mIU/ml). Monolayer cell number (lines) and protein content (bars) were determined at the indicated times. All data points and bars represent the mean ± SEM of triplicate cultures with the exception of the Day 6 protein values which are single observations.

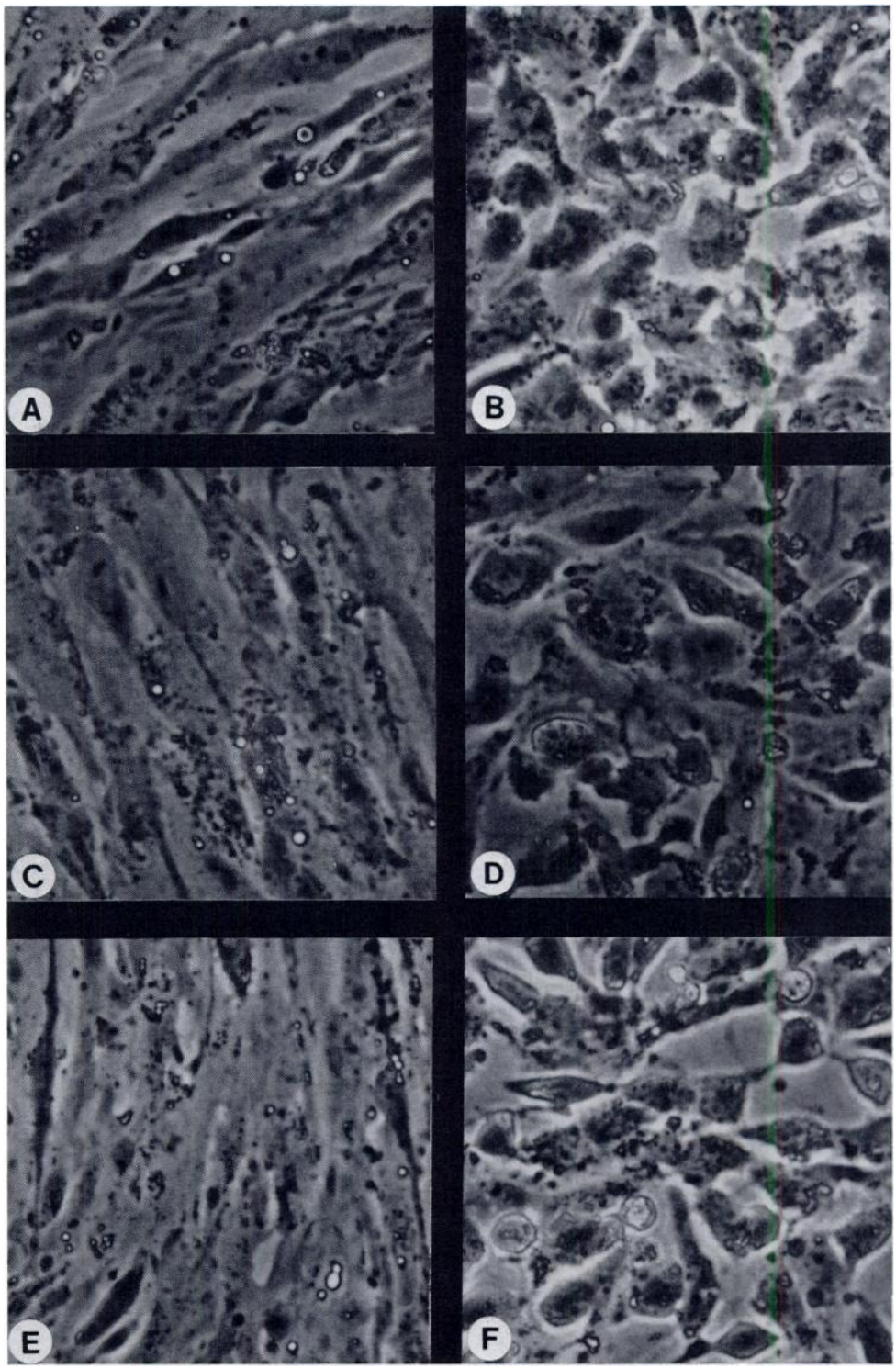

FIG. 4. Phase contrast photomicrographs illustrating the effect of insulin, FSH, and hCG on granulosa cell morphology. Cells were cultured for 3 days in medium alone A) or medium supplemented with the following: B) insulin, 25 mIU/ml; C) FSH, 30 ng/ml; D) insulin + FSH; E) hCG, 100 ng/ml; and F) insulin + hCG. ×470.

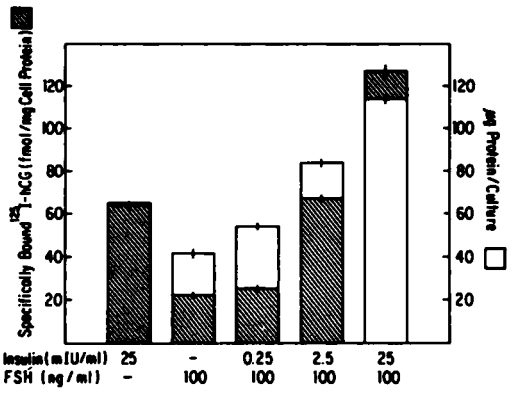

FIG. 5. Dose-response of FSH-mediated LH/hCG receptor induction with respect to insulin. Granulosa cells from 1–3 mm follicles were cultured for 6 days in medium supplemented with insulin (0–25 mIU/ml) and FSH (100 ng/ ml). The specific binding of [^{125}I] iodo-hCG was determined as described in the text, and normalized according to protein content. Data bars represent the mean ± SEM of three and five determinations for specific binding and protein, respectively.

further influenced by FSH or hCG (Fig. 4D, F, respectively).

Insulin Dose-Response for LH/hCG Receptor Induction

In our previous study (May et al., 1980) the extent of LH receptor induction was found to be dose-dependent with respect to FSH at a constant level of insulin (25 mIU/ml). Results presented in Fig. 5 indicate that FSH induction of LH/hCG receptor is also dose-dependent with respect to insulin. Receptor levels after 6 days of culture were significantly less ($P<0.001$) in monolayers treated with FSH alone (100 ng/ml) compared with those treated with insulin alone (25 mIU/ml). In combination with FSH, the lowest concentration of insulin tested (0.25 mIU/ml) had no effect; however, LH/hCG receptor levels were significantly and progressively increased by 2.5 and 25 mIU/ml insulin ($P<0.001$). Higher levels of insulin (125 and 250 mIU/ml) did not facilitate further increases in receptor induction relative to 25 mIU/ml (data not presented). It has been shown that insulin decreases the net loss of receptor in granulosa cell monolayers in the absence of FSH (May et al., 1980). Thus, LH/hCG receptor levels obtained at different concentrations of insulin may reflect both a protective or maintenance effect with regard to existing receptor and a facilitative effect with respect to the inductive action of FSH.

Effect of Insulin on Basal and Gonadotropin-Stimulated Progesterone Secretion

Granulosa cells maintained in serum-containing medium without insulin produced modest amounts of progesterone, the rate of which declined with time in culture (Figs. 6, 8). Insulin either maintained or slightly increased the basal level of steroid secretion during the culture period. As illustrated in Fig. 6, FSH alone elicited a dose-dependent stimulation of progesterone secretion which increased through Day 4 (2.1 – 2.9-fold increase relative to Day 2) but which declined dramatically through Day 6 (4.5–6.6-fold decrease relative to Day 4).

Insulin had little effect on FSH-stimulated progesterone production during the first 2 days of culture (Fig. 6); however, after Day 2, insulin consistently potentiated the steroidogenic response to FSH. During Days 2–4, the 10- and 17-fold increases in progesterone secretion (relative to Day 2 medium-alone controls) mediated by 10 and 100 ng/ml FSH, respectively, were potentiated by insulin to 16- and 33-fold, respectively. During Days 4–6 and relative to media controls, insulin enhanced the 6.8- and 15.5-fold increases in FSH-mediated progesterone secretion to 47- and 86-fold, respectively. In this study, total steroid secre-

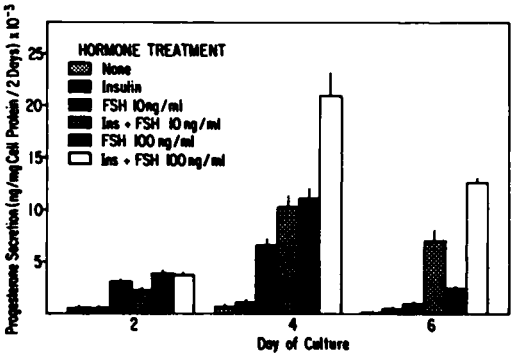

FIG. 6. Insulin effect on basal and FSH-stimulated progesterone secretion during culture. Granulosa cells were cultured as monolayers for 6 days in medium alone or medium supplemented with insulin (25 mIU/ml) and/or FSH (10 or 100 ng/ml). Each data bar represents the mean ± SEM of four samples.

tion declined during the last 2 days of culture. However, the loss of FSH responsiveness was considerably reduced in insulin-treated cells.

The progesterone secretory response to a constant level of FSH (100 ng/ml) was dose-dependent with respect to insulin (Fig. 7). Again, as previously noted, insulin had little effect during the first 2 days of culture. However, after 2 days, insulin concentrations of 2.5 and 25 mIU/ml significantly ($P<0.001$) potentiated FSH-stimulated progesterone secretion relative to FSH treatment alone.

Granulosa cells from 1–3 mm follicles were also tested for their steroidogenic response to hCG since it had been previously shown that these cells possess LH/hCG receptors (May et al., 1980). As indicated in Fig. 8, the cells responded in a manner similar to that observed with FSH alone (Fig. 6), i.e., maximal steroid secretion during Days 2–4 of culture followed by a decline in secretion during Days 4–6. Again, after the first 2 days of culture, insulin consistently potentiated hCG-stimulated progesterone secretion. In the presence of insulin, the low dose of hCG (10 ng/ml) elicited a linearly increased rate of steroid secretion throughout the culture period, whereas the higher dose (100 ng/ml) stimulated steroid secretion to a lesser extent and at a constant rate during Days 4–6. The effect of insulin relative to controls not treated with insulin was particularly evident during Days 4–6.

Aromatase Response to Insulin

An important biochemical marker of granulosa cell development is the production of estrogen. As originally determined for progesterone secretion, the ability of granulosa cell monolayers to secrete estrogen declines rapidly with time in culture (Haney and Schomberg, 1981). To determine the effect of insulin upon the maintenance of aromatase activity, granulosa cells were cultured for 6 days with combinations of insulin (25 mIU/ml), FSH (100 ng/ml), and testosterone (10^{-6} M). Spent media estrogen content was determined, and the results are presented in Fig. 9. Basal estrogen production declined with time in culture from 334 ± 43 pg/culture (mean ± SEM) during the first 2 days to levels which approximated those of the fresh culture medium (78.2 ± 25.7 pg/ml); the level and rate of decline were unaffected by various insulin/FSH combinations. The estrogen levels produced during the first 2 days may be a function of aromatase conversion of endogenous cellular androgen originally derived from the theca, or may represent de novo estrogen synthesis similar to that reported by McNatty et al. (1979) for human granulosa cells. In the presence of testosterone (Fig. 9, open bars), the granulosa cell monolayers secreted elevated levels of estrogen relative to nonsubstrate controls during the first 2 days of culture (9–10-fold increase, $P<0.001$). Insulin and/or FSH did

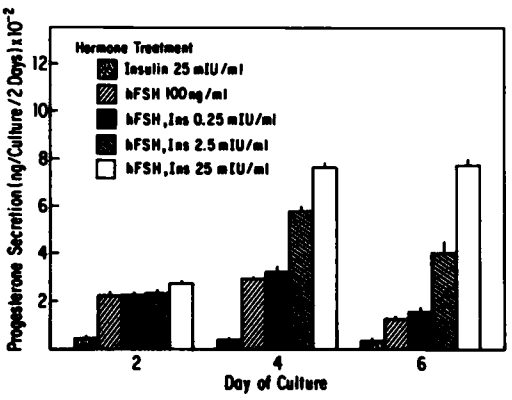

FIG. 7. Dose-response of FSH-stimulated steroidogenesis as a function of insulin. Data are derived from experimental media samples described in the legend of Fig. 5. Each bar represents the mean ± SEM of five samples assayed in duplicate.

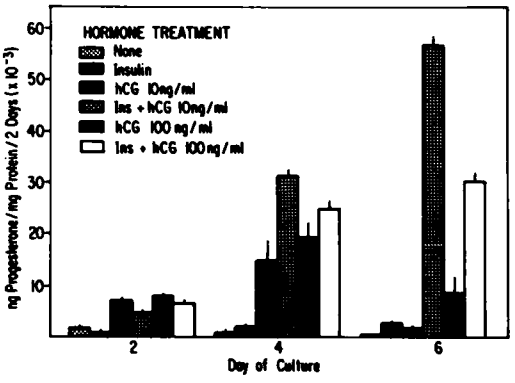

FIG. 8. Effect of insulin upon hCG-stimulated progesterone secretion during culture. Granulosa cells were cultured for 6 days in medium alone or medium supplemented with insulin (25 mIU/ml) and/or hCG (10 or 100 ng/ml). Each data bar represents the mean ± SEM of four replicate cultures assayed in duplicate.

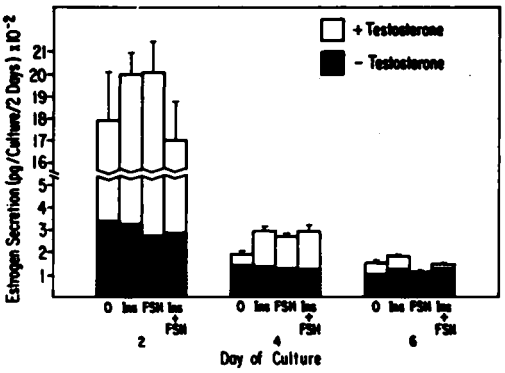

FIG. 9. Effect of insulin upon granulosa cell estrogen secretion during culture. Granulosa cells from 1–3 mm follicles were cultured as monolayers for 6 days in medium alone or in medium supplemented with insulin (25 mIU/ ml), FSH (100 ng/ml), or insulin + FSH. An identical set of cultures also contained 10^{-6} M testosterone. Each bar represents the mean ± SEM of four replicate cultures assayed in duplicate.

not affect estrogen secretion in the presence of testosterone, nor did they prevent the loss of aromatase activity. The decline in estrogen production cannot be ascribed to a generalized "dedifferentiation" of the culture system since progesterone production in these same cultures exhibited a typical pattern similar to that illustrated in Fig. 6 (data not presented). Thus, while insulin effectively facilitates and maintains cellular functions such as LH/hCG receptor induction and progesterone secretion, it fails to maintain at least one very important cellular biochemical parameter, estrogen production. It would appear that some specific component necessary for aromatase maintenance is lacking in the medium, or alternatively, perhaps a serum component is specifically inhibitory.

Specificity Studies

Relaxin is a polypeptide hormone primarily secreted by the corpus luteum of pregnancy, but may also be present in the ovary during the cycle (Bryant-Greenwood et al., 1980). It is chemically and structurally similar to insulin (reviewed by Schwabe et al., 1980). Although amino acid composition between these hormones yields only 40% homology, critical sequences determining tertiary structure appear to be conserved since relaxin fits the "insulin conformation" perfectly and without strain (Schwabe et al., 1980).

Because of the extreme degree of similarity between insulin and relaxin, it seemed logical to determine if this intraovarian insulin homologue could substitute for insulin as a determinant for LH/hCG receptor induction in the monolayer system. Granulosa cells were cultured under routine conditions with FSH (100 ng/ml) in combination with either insulin (25 mIU/ml) or highly purified porcine relaxin (10^1–10^4 ng/ml). After 6 days of culture, ^{125}I-hCG binding levels were compared with preculture levels. Unlike insulin, relaxin at all concentrations tested neither facilitated LH/hCG receptor induction nor maintained the preculture level of receptor (Fig. 10). When combined with insulin and FSH, relaxin failed to stimulate receptor induction beyond that of insulin and FSH alone (data not presented). Relaxin also failed to mimic insulin with regard to gonadotropin-stimulated progesterone secretion (data not presented).

Multiplication-stimulating activity (MSA) is a growth factor that has been isolated from serum (Pierson and Temin, 1972) and from the conditioned culture medium of Buffalo rat liver cells (BRL-3A; Dulak and Temin, 1973a,b). MSA has been shown to possess insulin-like activity in several systems (Nissley and Rechler, 1978; Salomon, 1980). MSA does not appear to compete for insulin receptors; however, insulin

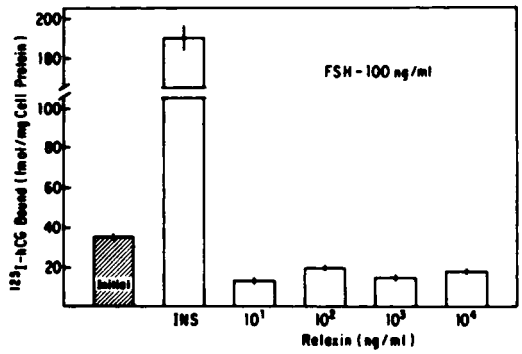

FIG. 10. Effect of relaxin on FSH-mediated LH/hCG receptor induction. Aliquots of freshly harvested granulosa cells were assayed for the initial level of [^{125}I] iodo-hCG binding. The remaining cells were cultured as monolayers in medium supplemented with FSH (100 ng/ml) and either insulin (25 mIU/ml) or porcine relaxin (1–1000 ng/ml). After 6 days of culture the [^{125}I] iodo-hCG binding capacity of the monolayers was determined. Each data bar represents the specific binding of triplicate determinations (mean ± SEM).

has been shown to interact with MSA receptors (Nissley and Rechler, 1978). To determine if the insulin effects in our system could be explained in part via interaction with putative MSA receptors, granulosa cells were cultured for 6 days with FSH (100 ng/ml) combined with either insulin (25 mIU/ml) or MSA (1–400 ng/ml). At the end of 6 days, monolayer ^{125}I-hCG binding was determined and compared with preculture levels. Unlike insulin, MSA was unable to facilitate receptor induction or prevent the loss of LH/hCG receptor (Fig. 11) normally observed in insulin-free cultures (May et al., 1980). The levels of MSA used were in accordance with those necessary to stimulate maximally [^{3}H] thymidine incorporation into chick embryo fibroblasts (Collaborative Research, MSA product specification sheet), a target cell that exhibits insulin cross reactivity with MSA receptors.

DISCUSSION

A major focus of attention in several laboratories, including our own, has been the establishment of model systems in vitro which reflect endocrine-dependent granulosa cell development in vivo, specifically LH/hCG receptor induction/function and steroidogenic competence. Rat and porcine granulosa cells have been used in the majority of studies. Erickson et al. (1979) and Orly et al. (1980) demonstrated convincingly that serum in vitro inhibits the responsiveness to gonadotropins of granulosa cells derived from DES-primed immature hypophysectomized rats. Porcine granulosa cells, however, appeared to be less sensitive to serum in that FSH-induction of the LH/hCG receptor could be demonstrated in vitro in serum-containing medium provided insulin was present (May et al., 1980). In this report we have presented evidence that insulin is critical to other aspects of granulosa cell development in vitro.

Channing et al. (1976) reported effects of insulin on immature porcine granulosa cells maintained under serum-free conditions. The plating efficiency achieved under these conditions was extremely low (<4%). Thus, it was not clear if the results were truly representative of granulosa cells in general, or rather, were reflective of a subpopulation of cells which was selected under serum-free conditions; in addition, many direct comparisons to serum-containing medium were not reported. Serum is known to facilitate cell attachment in vitro. This is reflected in our studies in which the plating efficiency for freshly harvested, viable granulosa cells was nearly 100%, a value consistent with that reported by Orly et al. (1980) for rat cells. It was suggested by Channing et al. (1976) that insulin might affect the plating efficiency of cells in serum-free medium. Our results indicated that in serum-containing medium insulin did not affect plating efficiency.

Unlike bovine granulosa cell cultures which exhibit a doubling time of 16 h in medium supplemented with 10% calf serum (Gospodarowicz et al., 1977), porcine granulosa cells grew slowly (Figs. 1–3), a finding consistent with that of Gospodarowicz and Bialecki (1979) and Orly et al. (1980) for porcine and rat granulosa cells, respectively. Insulin, which stimulates monolayer growth in several cell lines (Barnes and Sato, 1980; Mather and Sato, 1979), did not markedly stimulate division per se in porcine cells. Rather, insulin appeared to facilitate monolayer growth and cell maintenance as evidenced by a dramatic loss of cells in cultures deprived of insulin (Figs. 2, 3). Although monolayer growth and cell attachment during the early stages of culture were insulin-independent, further growth and maintenance were critically insulin-dependent. Part of the effect of insulin appeared to involve the establishment of an epithelioid cell morphology

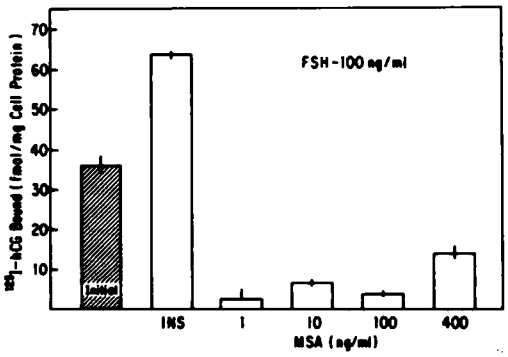

FIG. 11. Effect of multiplication-stimulating activity (MSA) on FSH-mediated LH/hCG receptor induction. Aliquots of freshly harvested granulosa cells were assayed for the initial level of [^{125}I] iodo-hCG binding. The remaining cells were cultured as monolayers in medium supplemented with FSH (100 ng/ml) and either insulin (25 mIU/ml) or MSA (1–400 ng/ml). After 6 days of culture, the [^{125}I] iodo-hCG binding capacity of the monolayers was determined. Each data bar represents the specific binding of triplicate determinations (mean ± SEM).

characteristic of granulosa cell differentiation in vivo. Immature, less differentiated cells from small follicles assumed a fibroblastic morphology in vitro (Fig. 4A) whereas mature cells from large follicles assume an epithelioid morphology (Channing, 1970). In immature granulosa cells in monolayer culture, insulin induced an epithelioid morphology which correlated with the ability of these cells to elicit differentiated functions, specifically, LH/hCG receptor induction and gonadotropin-stimulated steroidogenesis. FSH and hCG alone were incapable of eliciting the epithelioid morphology obtained with insulin and, furthermore, did not elicit nearly the same degree of cellular response as they did when combined with insulin.

Insulin enhancement of LH/hCG receptor induction and gonadotropin-stimulated progesterone secretion was not evident during the first 2 days of culture after which its effect became increasingly pronounced. Orly et al. (1980) did not observe an insulin effect on rat granulosa cell progesterone production, but this conclusion was based upon results obtained during the first 2 days of culture. Combined with the decline in LH/hCG receptor (May et al., 1980) the lack of insulin and gonadotropin responsiveness during the first 2 days of culture suggests that during this period the cells are limited quantitatively in their capacity to exhibit differentiated function. However, under the influence of insulin this capacity is subsequently expressed, resulting in an enhanced expression of differentiated function during moderate-term monolayer culture.

Insulin exerts a large number of diverse actions on cells; hence defining its mechanisms of action has been difficult. Insulin appears to act both at the level of the cell membrane, mediating short-term effects such as glucose and amino acid transport, and intracellularly, mediating delayed effects such as modulation of macromolecular synthesis and degradation (Goldfine, 1978). Thus, it is likely that the insulin effect on granulosa cells in vitro is the result of multiple actions. Insulin may exert a maintenance effect by enhancing the overall metabolic capacity of cells, allowing them to survive and function in culture. The delayed effect of insulin on receptor induction and steroidogenesis may be the result of an increased capacity of the cells to synthesize specific proteins, a decreased rate of protein degradation, or in the case of steroidogenesis, an increased enzymatic activity. The exact nature of the insulin effects, however, remains a matter of speculation.

Both LH/hCG receptor induction and gonadotropin-stimulated progesterone production were dose-dependent with respect to insulin. Mather and Sato (1979) reported that insulin is rapidly inactivated at 37°C in cysteine-containing nutrient media such as Ham's F-12 and Medium 199, presumably via interchain disulfide bond reduction. Although serum components may provide some protection via binding, the high levels of insulin required in vitro may in part be necessary to overcome sulfhydryl-mediated inactivation during culture. Microgram quantities of insulin are commonly used in vitro with several other cell types (Barnes and Sato, 1980; Florini and Roberts, 1979; Karl and Griswold, 1980). Rizzino and Sato (1978) have reported that media supplemented with 10% serum contains modest amounts of insulin (1 ng/ml). While such levels are likely to be found in our medium, they are clearly insufficient to support receptor induction. Insulin is known to interact with membrane receptors for somatomedin A, MSA, and nonsuppressible insulin-like activity (NSILA) in several systems (Nissley and Rechler, 1978). Thus, the insulin effects on granulosa cells may be due in part to insulin interaction with heterologous receptors, an explanation that would also justify the high concentrations of hormone required in vitro. However, our results demonstrating the inability of relaxin or MSA to substitute for insulin argue against this possibility.

Porcine granulosa cells maintained as monolayers in serum-containing medium supplemented with FSH alone lose LH/hCG receptors, express a limited progesterone secretory response to either FSH or hCG, and assume a fibroblastic morphology. These changes are consistent with dedifferentiation in vitro. Insulin counteracts this process, yielding a responsive system in vitro which is more reflective of granulosa cell development in vivo in terms of LH/hCG receptor content, gonadotropin responsiveness, and morphologic development. Thus, insulin should be considered for routine use during moderate-term monolayer culture of porcine granulosa cells.

ACKNOWLEDGMENTS

The authors wish to acknowledge Dr. O. David Sherwood of the University of Illinois for his material support and guidance in the studies dealing with relaxin.

The authors also wish to acknowledge the invaluable technical assistance of Anne Brown, Sherry Bass, and Rebecca Addison. We also wish to thank Donna Sanders for her secretarial assistance.

REFERENCES

Aksel, S., Schomberg, D. W., Tyrey, L., Hammond, C. B. (1976). Vasomotor symptoms, serum estrogens, and gonadotropin levels in surgical menopause. Am. J. Obstet. Gynecol. 125, 165–169.

Barnes, D. and Sato, G. (1980). Methods for growth of cultured cells in serum-free medium. Anal. Biochem. 102, 255– 270.

Bryant-Greenwood, G. D., Jeffrey, R., Ralph, M. M., Seamark, R. F. (1980). Relaxin production by the porcine ovarian Graafian follicle in vitro. Biol. Reprod. 23, 792–800.

Channing, C. P. (1970). Influences of the *in vivo* and *in vitro* hormonal environment upon luteinization of granulosa cells in tissue culture. Rec. Prog. Horm. Res. 26, 589–622.

Channing, C. P., Tsai, V. and Sachs, D. (1976). Role of insulin, thyroxin and cortisol in luteinization of porcine granulosa cells in chemically defined media. Biol. Reprod. 15, 235–247.

Dulak, N. C. and Temin, H. M. (1973a). A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. J. Cell. Physiol. 81, 153–160.

Dulak, N. C. and Temin, H. M. (1973b). Multiplication-stimulating activity for chicken embryo fibroblasts from rat liver cell conditioned medium: A family of small polypeptides. J. Cell. Physiol. 81, 161–170.

Erickson, G. F., Wang, C., Hsueh, A.J.W. (1979). FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium. Nature 279, 336–338.

Florini, J. R. and Roberts, S. B. (1979). A serum-free medium for the growth of muscle cells in culture. In Vitro 15, 983–992.

Goldfine, I. D. (1978). Insulin receptors and the site of insulin action. Life Sci. 23, 2639–2648.

Gospodarowicz, D., Ill, C. R. and Birdwell, C. R. (1977). Effects of fibroblast and epidermal growth factors on ovarian cell proliferation *in vitro*. 1. Characterization of the response of granulosa cells to FGF and EGF. Endocrinology 100, 1108–1120.

Gospodarowicz, D. and Bialecki, H. (1979). Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine, and human origin. Endocrinology 104, 757–764.

Ham, R. G. and McKeehan, W. L. (1979). Media and growth requirements. Methods Enzymol. 58, 44–93.

Haney, A. F. and Schomberg, D. W. (1978). Steroidal modulation of progesterone secretion by granulosa cells from large porcine follicles: A role for androgens and estrogens in controlling steroidogenesis. Biol. Reprod. 19, 242–248.

Haney, A. F. and Schomberg, D. W. (1981). Estrogen and progesterone production by developing porcine follicles in vitro: Evidence for estrogen formation by theca. Endocrinology. In Press.

Hayashi, I., Larner, J. and Stato, G. (1978). Hormonal growth control of cells in culture. In Vitro 14, 23–30.

Karl, A. F. and Griswold, M. D. (1980). Actions of insulin and vitamin A on Sertoli cells. Biochem. J. 186, 1001–1003.

Mather, J. P. and Sato, G. H. (1979). The growth of mouse melanoma cells in hormone-supplemented, serum-free medium. Exp. Cell. Res. 120, 191–200.

May, J. V., McCarty, K., Jr., Reichert, L. E., Jr. and Schomberg, D. W. (1980). Follicle-stimulating hormone-mediated induction of functional luteinizing hormone/human chorionic gonadotropin receptors during monolayer culture of porcine granulosa cells. Endocrinology 107, 1041–1047.

McNatty, K. P., Makris, A., DeGrazia, C., Osathanondh, R. and Ryan, K. (1979). The production of progesterone, androgens, and estrogens by granulosa cells, thecal tissue, and stromal tissue from human ovaries, *in vitro*. Endocrinology 49, 687–699.

Mondschein, J. S. and Schomberg, D. W. (1981). Growth factors modulate gonadotropin receptor induction in granulosa cell cultures. Science 211, 1179–1180.

Nimrod, A., Tsafriri, A. and Lindner, H. R. (1977). *In vitro* induction of binding sites for hCG in rat granulosa cells by FSH. Nature 267, 632–633.

Nissley, S. P. and Rechler, M. M. (1976). Multiplication-stimulating activity (MSA): A somatomedin-like polypeptide from cultured rat liver cells. In: Third Decennial Review Conference: Cell, Tissue, and Organ Culture. Gene Expression and Regulation in Cultured Cells. (K. K. Sanford, ed.). Natl. Cancer Inst. Monogr. 48, 167–177.

Orly, J., Sato, G. and Erickson, G. F. (1980). Serum suppresses the expression of hormonally induced functions in cultured granulosa cells. Cell 20, 817–827.

Pierson, R. W., Jr. and Temin, H. M. (1972). The partial purification from calf serum of a fraction with multiplication-stimulating activity for chicken fibroblasts in cell culture and with non-suppressible insulin-like activity. J. Cell. Physiol. 79, 319–330.

Rizzino, A. and Sato, G. (1978). Growth of embryonal carcinoma cells in serum-free medium. Proc. Natl. Acad. Sci. 75, 1844–1848.

Rizzino, A. and Sato, G. (1978). Growth of embryonal carcinoma cells in serum-free medium. Proc. Nat'l. Acad. Sci. 75, 1844–1848.

Salomon, D. S. (1980). Correlation of receptors for growth factors on mouse embryonal carcinoma cells with growth in serum-free hormone-supplemented medium. Exp. Cell Res. 128, 311–321.

Schwabe, C., Steinetz, B., Weiss, G., Segaloff, A., McDonald, J. K., O'Byrne, E., Hochman, K., Carriere, B. and Goldsmith, L. (1978). Relaxin. Rec. Prog. Horm. Res. 34, 123–211.

Stouffer, R. L., Tyrey, L., Schomberg, D. W. (1976). Changes in [^{125}I]labeled human chorionic gonadotropin (hCG) binding to porcine granulosa cells during follicle development and cell culture. Endocrinology 99, 516–525.