BIOLOGY OF REPRODUCTION 40, 735–743 (1989)

Insulin Enhances Luteinizing Hormone-Stimulated Steroidogenesis by Porcine Theca Cells[1]

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ABSTRACT

It has been shown recently that insulin enhances differentiation of rat, pig, and human granulosa cells. The present studies were done to determine if insulin also plays a role in the regulation of theca cell steroidogenesis. Theca cells were obtained from prepubertal gilts and cultured under serum-free conditions for 48 h. Theca cell androstenedione production under basal and luteinizing hormone (LH)-stimulated conditions was significantly increased by adding insulin (1 μg/ml) to the culture medium. Treatment of basal and LH-stimulated cultures with increasing concentrations of insulin (0.001–10 μg/ml) caused dose- and time-dependent increments in androstenedione production, but the effect was independent of the dose of LH employed. The ability of insulin to enhance thecal cell androstenedione production was mimicked by somatomedin C, but not by relaxin.

Studies to determine the mechanism(s) of action of insulin showed that insulin action is exerted, at least in part, at a site(s) proximal to cyclic adenosine 3'5'-monophosphate (cAMP) generation, since insulin enhanced both basal and LH-stimulated accumulation of extracellular cAMP in addition to increasing androstenedione production. This effect was further enhanced by 3-isobutyl-1-methyl xanthine, an inhibitor of phosphodiesterase activity. Insulin treatment also caused dose-dependent increments in forskolin- and prostaglandin E_2-stimulated accumulation of extracellular cAMP and androstenedione. Insulin also increased both the basal and LH-stimulated production of progesterone and its precursor pregnenolone, in addition to the increases in androstenedione. Furthermore, insulin enhanced the ability of theca cells to produce androstenedione in response to an exogenously supplied sterol substrate 25-hydroxycholesterol, and to exogenous pregnenolone.

These experiments show that insulin causes significant stimulatory effects on porcine theca cell steroidogenesis, at least in part via an enhancement of the generation of cAMP, leading to increased activity of the steroidogenic enzymes cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase. These results indicate that insulin, or insulin-like growth factors, may play an important role in the regulation of follicular development, since thecal androgens are the substrates for granulosa cell estrogen biosynthesis and are also involved in follicular atresia.

INTRODUCTION

Theca cells in the pig ovary synthesize and secrete androgens, which play a key role in estrogen biosynthesis by granulosa cells (Armstrong and Dorrington, 1977) and possibly play a role in follicular atresia (Louvet et al., 1975). Although it is widely accepted that the pituitary gonadotropin luteinizing hormone (LH) provides the primary control of theca cell steroidogenesis (Baird and McNeilly, 1981), other circulating

Accepted November 16, 1988.
Received August 5, 1988.
[1]This work was supported by grants from the Medical Research Council of Canada (Group grant to D.T.A. and MT 2096 to F.R.C.). D. T. Armstrong is a Career Investigator of the MRC. P. Morley was supported by an MRC Studentship.
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and follicular fluid factors can modify the responsiveness of theca cells to LH. For example, estradiol-17β (Leung and Armstrong, 1979a,b), prolactin (Magoffin and Erickson, 1982), epidermal growth factor (Erickson and Case, 1983), and gonadotropin-releasing hormone (Magoffin et al., 1981) have been shown to inhibit theca cell androgen production. On the other hand, lipoproteins (Dyer et al., 1985), catecholamines (Dyer and Erickson, 1985), insulin (Barbieri et al., 1983; Erickson and Case, 1983; Erickson et al., 1985), and insulin-like growth factors (Caubo and Tonetta, 1988; Hernandez et al., 1988) have been shown to enhance theca cell androgen production.

The effects and mechanism of action of insulin on follicular steroidogenesis have been studied extensively using granulosa cells. Specific insulin receptors are present on porcine granulosa cells (Rein and Schomberg, 1982) and are functionally coupled to cellular responses. The addition of insulin to cultured porcine granulosa cells has been shown to induce trophic effects (Veldhuis et al., 1983) and induces differentiative changes characterized by altered cell morphology and increased rates of steroid hormone biosynthesis (Channing et al., 1976; May and Schomberg, 1981; Veldhuis et al., 1983). These effects have been shown to involve the induction of steroidogenic enzymes (Veldhuis et al., 1983; Davoren and Hsueh, 1984).

The actions of insulin on theca cell function were reported by Barbieri et al. (1983), who showed that insulin augmented LH-stimulated androgen production by cultured porcine theca tissue, and by Erickson and Case (1983), who demonstrated that insulin significantly increased LH-stimulated androgen production by cultured rat theca-interstitial cells. However, the site and mechanism of action of these stimulatory effects of insulin are not known. Therefore, the present study was designed to investigate the effects and the mechanism(s) of action of insulin on theca cell steroidogenesis.

MATERIALS AND METHODS

Porcine Theca Cell Cultures

Theca cells were obtained from the ovaries of prepubertal gilts as described by Tsang et al. (1979) and modified by Hunter and Armstrong (1987). Briefly, ovaries were obtained at a local abattoir (Thorndale Abattoir, Thorndale, Ontario) and transported to the laboratory on ice. Follicles of 3–6 mm diameter were cut in half and the granulosa cells were scraped off. The theca interna was separated mechanically from the theca externa and dispersed enzymatically with 0.25% collagenase (Type II, Sigma Chemical Co., St. Louis, MO), 0.05% hyaluronidase (Type I, Sigma), and 0.05% protease (Type XIV, Sigma). Cultures consisted of 500,000 theca cells in 1 ml medium (Gibco Laboratories, Grand Island, NY) plated into each well in 24-well tissue culture plates (Falcon Plastics, Los Angeles, CA). Culture medium was Dulbecco's Modified Eagle's medium containing 1.2 g/l $NaHCO_3$ plus antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin, and 0.625 μg/ml Fungizone). Incubations were carried out at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Effects of insulin on steroid production were studied in the absence and presence of a maximally stimulating dose of LH.

Reagents and Hormones

LH used in these studies was USDA-bovine LH B5. Bovine insulin was obtained from Collaborative Research, Bedford, MA. 25-Hydroxycholesterol was purchased from Steraloids, Wilton, NH. Prostaglandin E_2 (PGE_2) was obtained from The Upjohn Co., Kalamazoo, MI. Somatomedin-C (Sm-C) was obtained from Amersham Corp., Arlington Heights, IL. Porcine relaxin was obtained from the NIH, Bethesda, MD. Forskolin was obtained from Calbiochem-Behring, La Jolla, CA. Pregnenolone and 3-isobutyl-1-methyl xanthine (MIX) were purchased from Sigma.

Radioimmunoassay of Secreted Products

In all studies except the time course study, media were removed after 48 h of incubation and stored at –20°C until assayed for secreted androstenedione (Leung and Armstrong, 1979a), progesterone (Leung and Armstrong, 1979b), and estradiol-17β (Daniel and Armstrong, 1984) using radioimmunoassays that have been previously described and validated for direct measurements in culture media. A new radioimmunoassay was developed for pregnenolone using [7-^{3}H]pregnenolone (New England Nuclear, Dorval, Quebec) as a radioligand and an antibody that had been raised in a rabbit against pregnenolone-16α-carboxymethyl bovine serum albumin according to the method of Inaba et al. (1979). The antiserum was used at a working dilution at 1:3000, which produced an initial

TABLE 1. Cross-reactivities of antiserum to pregnenolone.

Steroid	Percent cross-reaction*
Pregnenolone	100. %
Cholesterol	2.55
5α-Pregnane-3β,20α-diol	2.18
Progesterone	0.14
20α-Hydroxypregn-4-en-3-one	0.096
5β-Pregnane-3,20-dione	<0.01
20β-Hydroxypregn-4-en-3-one	<0.01
5α-Pregnane-3,20-dione	<0.01
17α-Hydroxypregn-4-en-3,20-dione	<0.01
5α-Pregnan-3α-ol-20-one	<0.01
Testosterone	<0.01
Estrone	<0.01
Estradiol-17β	<0.01
Estriol	<0.01
Androstenedione	<0.01
19-Nortestosterone	<0.01

*Based on relative amounts required to inhibit the binding of [^{3}H]pregnenolone by 50%.

binding of radioligand that was typically 63.9%. The range of the standard curve was from 6.25 to 3200 pg. The calculated minimum detectable dose was typically 12.3 pg. The within-assay coefficient of variation determined by direct assay of six replicate measurements from the same sample (151 pg/100 μl) was 7.3%. The between-assay coefficient of variation was 14.4%. The relative cross-reactivities of this antiserum are listed in Table 1. Validation consisted of the following procedures. Direct assay on medium agreed with assay after extraction with diethyl ether following correction for recovery (92.1%). Parallelism was confirmed between the standard curve and a range of experimental samples assayed directly in volumes of medium ranging from 5 to 100 μl. For the determination of cyclic adenosine 3′5′-monophosphate (cAMP), 0.2 ml medium was withdrawn from cultures, heated in a boiling water bath for 20 min, and stored at –20°C until assayed by a previously validated radioimmunoassay (Reddoch and Armstrong, 1984).

Statistical Analyses

Statistical comparisons were made by analysis of variance. When significant effects were observed, Duncan's new multiple range test was used for multiple comparisons. Treatments were replicated in quadruplicate within an experiment, and each experiment was performed 2–3 times. All tests were performed as described by Steel and Torrie (1960), and statistical significance was inferred by $p<0.05$.

RESULTS

Effect of Insulin and Sm-C on Theca Cell Androstenedione Production

To investigate the dose-dependence of the effects of insulin and Sm-C on basal and LH-stimulated androstenedione production, theca cells were cultured in the absence or presence of LH (250 ng/ml) with or without increasing concentrations (0.0001–10 μg/ml) of insulin or Sm-C (0.1–100 ng/ml) (Figs. 1A and B). Treatment with increasing concentrations of either insulin or Sm-C resulted in dose-dependent increments in basal and LH-stimulated androstenedione production. The results presented in Figures 1A and 1B were obtained from different theca cell preparations, hence the amount of androstenedione production in the absence or insulin and Sm-C are different. The effects of insulin and Sm-C on theca cell androstenedione production were not mimicked by the structurally related peptide relaxin (0.001–10 μg/ml; data not shown).

To examine the effect of increasing concentrations of LH on the ability of insulin to stimulate theca cell androstenedione production, theca cells were cultured in the absence or presence of increasing concentrations of LH (31.25–500 ng/ml) with or without a maximally stimulating dose of insulin (5 μg/ml). Treatment with increasing concentrations of LH resulted in dose-de-

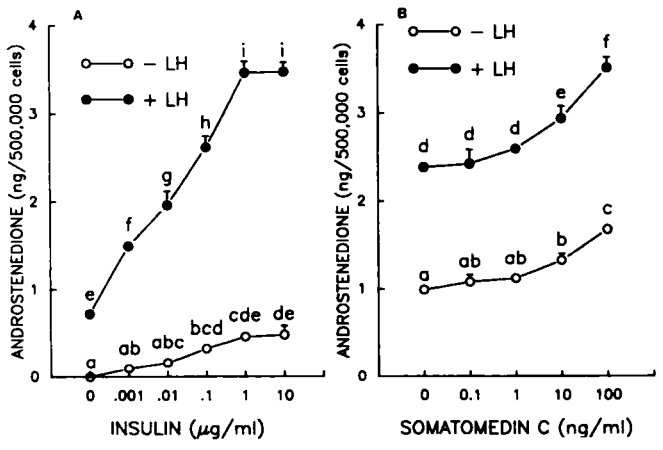

FIG. 1. Concentration-dependence of the effects of insulin (*A*) and somatomedin-C (*B*) on theca cell androstenedione production. Theca cells were incubated without luteinizing hormone (-LH) or with 250 ng/ml LH (+LH) in the absence or presence or increasing concentrations (0.0001–10 μg/ml) of insulin or somatomedin-C (0.1–100 ng/ml). Values represent the mean ± SEM of quadruplicate cultures from a typical experiment. Values with different *superscripts* are significantly different ($p<0.05$). Insulin and somatomedin-C increased both basal and LH-stimulated androstenedione production.

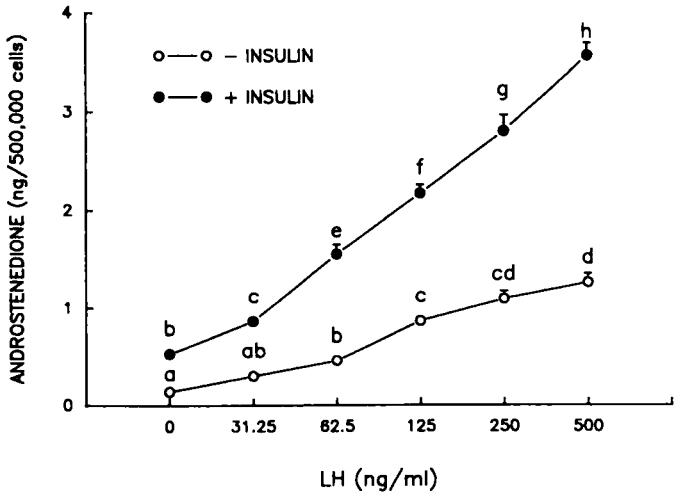

FIG. 2. Effect of insulin on luteinizing hormone (LH) dose-dependent stimulation of androstenedione production by porcine theca cells. Theca cells were cultured in the absence or presence of increasing concentrations of LH (31.25–500 ng/ml), with or without a maximally stimulating dose of insulin (5 μg/ml). Values represent the mean ± SEM of quadruplicate cultures from a typical experiment. Values with different *superscripts* are significantly different ($p<0.05$). Insulin significantly increased androstenedione production at all levels of LH stimulation.

pendent increments in androstenedione production in the absence and presence of insulin (Fig. 2). In the presence of insulin, androstenedione production was stimulated at least 2.5-fold at every dose of LH tested, and a dose of LH (31.25 ng/ml) that by itself was not capable of stimulating androstenedione production was rendered effective by the addition of insulin, eliciting a response comparable to that induced by a dose of 125 ng/ml LH. Analysis of variance revealed a significant ($p<0.05$) interaction between LH concentration and insulin treatment.

To investigate the time course of insulin-enhanced androstenedione production, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without a maximally stimulating dose of insulin (5 μg/ml) for varying incubation times (6–48 h). Time-dependent increments in androstenedione production were observed in both basal and LH-stimulated cultures in the absence and presence of insulin. Treatment with insulin caused significant ($p<0.05$) increases in androstenedione production under both basal and LH-stimulated conditions at all times greater than 6 h. These results are shown in Figure 3.

Influence of Insulin on LH-, Forskolin-, PGE_2-, and MIX-Induced Accumulation of Extracellular cAMP and Androstenedione by Porcine Theca Cells

To evaluate the effect of treatment with insulin on basal and LH-stimulated accumulation of extracellular cAMP, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without insulin at a concentration of 5 μg/ml (Table 2). Treatment with insulin resulted in a significant ($p<0.05$) increase in both basal and LH-stimulated accumulation of extracellular cAMP. The addition of the phosphodiesterase inhibitor, MIX (400 μM), enhanced the overall accumulation of cAMP in all treatment groups. Androstenedione levels were measured in the same cultures (Table 2). Treatment with insulin resulted in a significant ($p<0.05$) increase in both basal and LH-stimulated androstenedione production, and in the presence of MIX, the overall accumulation of androstenedione was further enhanced in all treatment groups. Analysis of variance revealed significant ($p<0.05$) interactions between LH

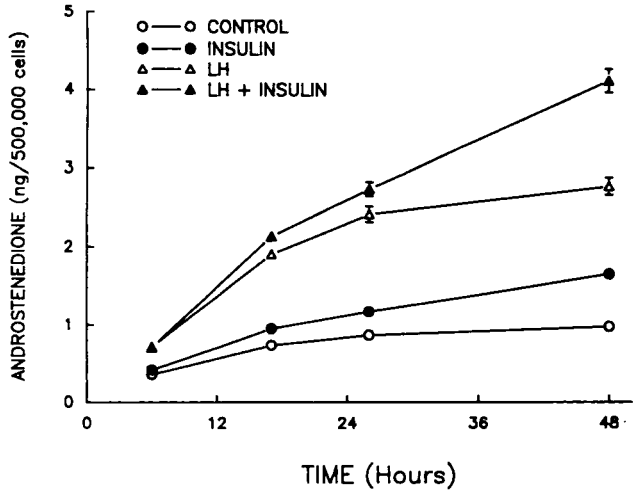

FIG. 3. Time course of insulin stimulation of porcine theca cell androstenedione production. Theca cells were cultured in the absence or presence of luteinizing hormone (LH, 250 ng/ml), with or without a maximally stimulating dose of insulin (5 μg/ml). The medium was collected at varying times (6–48 h) after plating. Values represent the mean ± SEM of quadruplicate cultures from a typical experiment. Values with different *superscripts* are significantly different ($p<0.05$). Insulin significantly increased androstenedione production under basal and LH-stimulated conditions at all time points greater than 6 h.

TABLE 2. Effect of insulin, in the absence and presence of a phosphodiesterase inhibitor (MIX) on basal and luteinizing hormone (LH)-stimulated accumulation of extracellular cyclic adenosine 3',5'-monophosphate (cAMP) and androstenedione by porcine theca cells.

Treatments*	Extracellular cAMP** (pmol/500,000 cells)	Androstenedione** (ng/500,000 cells)
CON	1.35 ± 0.11	6.23 ± 0.62
CON + MIX	5.09 ± 0.37	13.80 ± 0.40
INS	3.50 ± 0.28	11.48 ± 0.60
INS + MIX	7.88 ± 0.63	22.12 ± 0.50
LH	23.51 ± 1.35	14.16 ± 0.51
LH + MIX	107.02 ± 5.17	24.47 ± 2.03
LH + INS	38.02 ± 1.96	23.19 ± 2.07
LH + INS + MIX	127.64 ± 6.79	38.07 ± 3.17

*Theca cells were cultured in the absence (CON) or presence (LH) of LH (250 ng/ml), with or without insulin (INS; 5 μg/ml); some of the cultures contained MIX (400 μM).

**Values represent the mean ± SEM of quadruplicate cultures from a typical experiment; analysis of variance revealed significant ($p<0.05$) interactions between LH and MIX and LH and insulin.

and MIX and LH and insulin for extracellular cAMP and androstenedione accumulation.

To study the ability of insulin to enhance the action of other pharmacological and physiological agonists, for which cAMP is a second messenger, theca cells were cultured in the absence or presence of increasing concentrations (0.1, 1.0, and 10 μg/ml) of PGE_2 or forskolin (10^{-6}, 10^{-5}, and 10^{-4} M), with or without insulin at a concentration of 5 μg/ml. Treatment with increasing concentrations of PGE_2 resulted in dose-dependent increments in cAMP (Fig. 4A) and androstenedione (Fig. 4B) accumulation, and insulin enhanced the stimulatory effects of PGE_2 at all doses tested. Similar effects of insulin were observed when forskolin was the adenylate cyclase-stimulating agent employed (Figs. 5A and 5B).

Effect of Insulin on Pregnenolone and Progesterone Production and Conversion of Exogenous 25-Hydroxycholesterol and Pregnenolone to Androstenedione by Porcine Thecal Cells

To investigate the site(s) in the steroidogenic pathway at which insulin and LH exerted their stimulatory effects, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without increasing concentrations (0.001–10 μg/ml) of insulin, and the steroids pregnenolone, progesterone, and androstenedione were measured at the end of 48 h of culture. As shown in Table 3, cultured porcine theca cells produced significant amounts of pregnenolone and progesterone, in addition to androstenedione. Treatment with increasing concentrations of insulin resulted in dose-dependent increments in basal and LH-stimulated pregnenolone and progesterone production. The elevation of pregnenolone production in the presence of insulin suggests that the effects of insulin on theca cell steroidogenesis are expressed at a site(s) prior to the production of pregnenolone.

To further investigate the possible site of insulin action, an experiment was done to determine if insulin could affect the ability of theca cells to convert a soluble sterol substrate for the cholesterol side-chain cleavage enzyme, 25-hydroxycholesterol, to androstenedione. Theca cells were cultured in the absence or

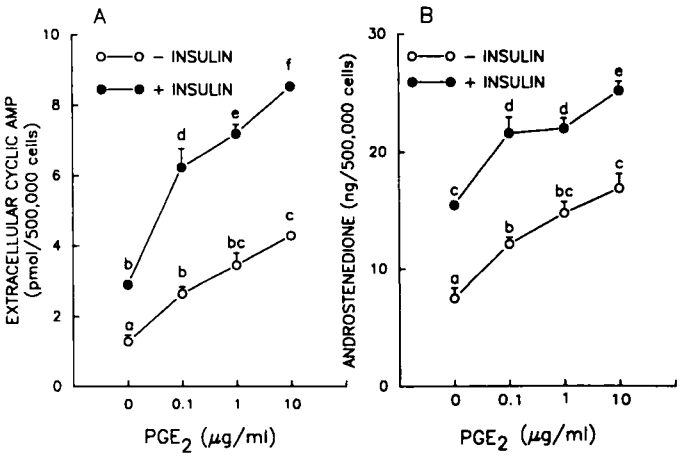

FIG. 4. Effect of insulin on prostaglandin E_2 (PGE_2)-stimulated extracellular cyclic adenosine 3'5'-monophosphate (cAMP) (A) and androstenedione (B) accumulation by porcine theca cells. Theca cells were incubated in the absence or presence of increasing concentrations (0.1, 1.0, and 10 μg/ml) or PGE_2, with or without a maximally stimulating dose of insulin (5 μg/ml). Values represent the mean ± SEM of quadruplicate cultures from a typical experiment. Values with different *superscripts* are significantly different ($p<0.05$). Insulin significantly increased PGE_2-stimulated extracellular cAMP and androstenedione accumulation.

TABLE 3. Effect of insulin on the production of pregnenolone, progesterone, and androstenedione by porcine theca cells.

Treatment*	Steroid production (ng/500,000 cells)**		
	Androstenedione	Progesterone	Pregnenolone
Control	0 ± 0[a]	8.61 ± 0.74[a]	0.09 ± 0.07[a]
Insulin (μg)			
0.001	0.10 ± 0.03[ab]	11.09 ± 0.66[a]	0.04 ± 0.01[a]
0.01	0.17 ± 0.03[abc]	12.74 ± 0.69[a]	0.05 ± 0.02[a]
0.1	0.33 ± 0.05[bcd]	35.23 ± 1.14[b]	0.31 ± 0.08[ab]
1.0	0.47 ± 0.06[cde]	48.19 ± 4.30[c]	0.69 ± 0.13[bc]
10.0	0.49 ± 0.10[de]	55.15 ± 1.71[c]	0.71 ± 0.11[bc]
LH	0.72 ± 0.05[e]	14.47 ± 1.04[a]	0.38 ± 0.04[ab]
LH + Insulin (μg)			
0.001	1.50 ± 0.04[f]	32.39 ± 2.26[b]	0.40 ± 0.05[ab]
0.01	1.96 ± 0.16[g]	55.86 ± 2.79[c]	1.02 ± 0.05[c]
0.1	2.61 ± 0.13[h]	108.26 ± 5.59[d]	1.65 ± 0.12[d]
1.0	3.46 ± 0.13[i]	126.46 ± 3.71[e]	2.23 ± 0.16[e]
10.0	3.47 ± 0.11[i]	143.16 ± 4.00[f]	2.36 ± 0.35[e]

*Theca cells were cultured with (LH) or without (Control) luteinizing hormone (250 ng/ml) in the absence or presence of increasing concentrations (0.001–10 μg/ml) of insulin.

**Values represent the mean ± SEM of quadruplicate cultures from a typical experiment; values with different superscripts are significantly different ($p<0.05$).

presence of LH (250 ng/ml), with or without 25-hydroxycholesterol (50 μg/ml). Some cultures contained a maximally stimulating dose of insulin (5 μg/ml). Figure 6 shows that LH-stimulated theca cells respond to 25-hydroxycholesterol with a significant ($p<0.05$) increase in androstenedione production. Insulin significantly ($p<0.05$) increased progesterone production in the absence and presence of 25-hydroxycholesterol. Cultures without LH responded in a qualitatively similar manner to cultures with LH, but the absolute levels of steroid production were lower (data not shown). In a similar experiment, we investigated whether insulin could affect the ability of theca cells to convert exogenous pregnenolone, a substrate for the 3β-hydroxysteroid dehydrogenase enzyme, to androstenedione. Theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without increasing concentrations

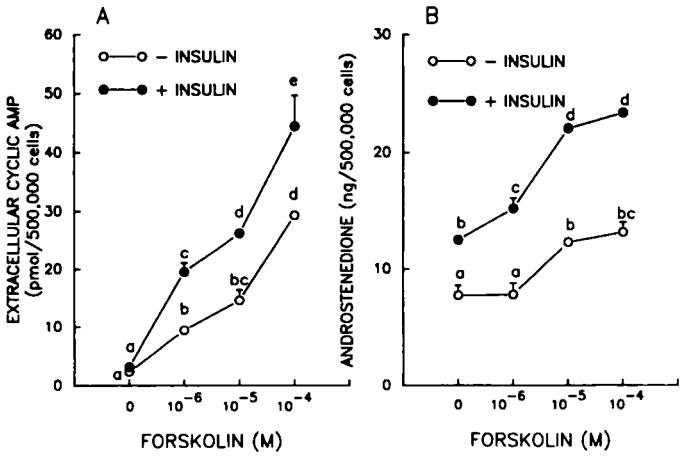

FIG. 5. Effect of insulin on *forskolin*-stimulated extracellular cyclic adenosine 3′5′-monophosphate (cAMP) *(A)* and androstenedione *(B)* production by porcine theca cells. Theca cells were cultured in the absence or presence of increasing concentrations (10^{-6}, 10^{-5}, and 10^{-4} M) of forskolin, with or without a maximally stimulating dose of insulin (5 μg/ml). Values represent the mean ± SEM of quadruplicate cultures from a typical experiment. Values with different *superscripts* are significantly different ($p<0.05$). Insulin significantly increased forskolin-stimulated extracellular cAMP and androstenedione accumulation.

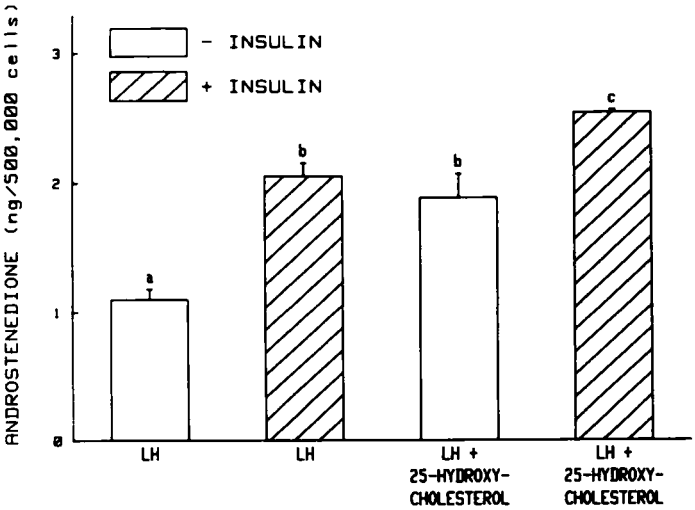

FIG. 6. Effect of insulin (5 μg/ml) on the conversion of exogenous 25-hydroxycholesterol to androstenedione by theca cells. Theca cells were cultured in the presence of luteinizing hormone (LH, 250 ng/ml), with or without 25-hydroxycholesterol (50 μg/ml). Values are the mean ± SEM of quadruplicate cultures from a typical experiment. Values with different *superscripts* are significantly different ($p<0.05$). Insulin alone significantly stimulated androstenedione production over control levels and caused further significant increases in androstenedione production in the presence of 25-hydroxycholesterol.

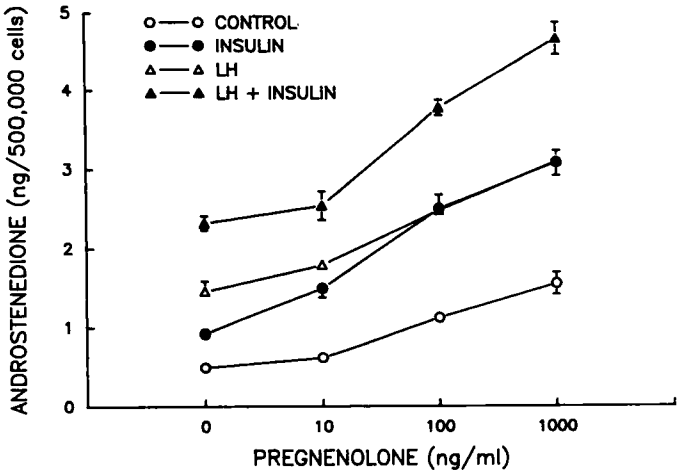

FIG. 7. Effect of insulin on the conversion of exogenous pregnenolone to androstenedione by theca cells. Theca cells were cultured in the absence or presence of luteinizing hormone (LH, 250 ng/ml), with or without increasing concentrations (10–1000 ng/ml) of pregnenolone. Some cultures contained a maximally stimulating dose of insulin (5 μg/ml). Values are the mean ± SEM of quadruplicate cultures from a typical experiment. Values with different *superscripts* are significantly different ($p<0.05$). Increasing concentrations of pregnenolone caused significant dose-dependent increments in androstenedione production in basal and LH-stimulated cultures. Insulin caused further significant increases in androstenedione production at all concentrations of pregnenolone tested.

(10–10,000 ng/ml) of exogenously supplied pregnenolone. Theca cells were cultured with or without a maximally stimulating concentration of insulin (5 μg/ml). Treatment with increasing concentrations of pregnenolone produced significant ($p<0.05$) increases in theca cell androstenedione production in both basal and lH-stimulated cultures (Fig. 7). In the presence of insulin, further significant ($p<0.05$) increases in androstenedione production were observed.

DISCUSSION

The role of insulin as a factor that can influence theca cell steroidogenesis was investigated with porcine theca cells. Our findings indicate that insulin can enhance both basal and LH-stimulated theca cell steroidogenesis. The insulin effect was dose- and time-dependent, but was independent of the LH dose employed. These results are in agreement with those of Barbieri et al. (1983) and Caubo and Tonetta (1988) from studies using cultured porcine theca cells. In the rat theca-interstitial cell preparation, however, insulin enhances LH-stimulated steroidogenesis, but has no effect alone (Erickson and Case, 1983; Erickson et al., 1985). Previous reports of the effects of insulin and/or Sm-C on rat, pig, and human granulosa cell steroidogenesis have also shown that insulin is inactive by itself but acts synergistically with FSH to stimulate progesterone production (Baranao and Hammond, 1984; Davoren and Hsueh, 1984; Adashi et al., 1985a; Veldhuis et al., 1985a), as well as the induction of LH receptors (Adashi et al., 1985b) and augmentation of aromatase activity (Garzo and Dorrington, 1984; Adashi et al., 1985c). This augmentation is not FSH-specific, but can be obtained with other substances that stimulate granulosa cells, for which cAMP is a second messenger (Adashi et al., 1986). In cultured porcine granulosa cells, there are conflicting results regarding whether insulin does (Veldhuis et al., 1985a) or does not (Baranao and Hammond, 1984) exert independent effects. The reasons for these discrepancies are not clear, but the discrepancies may be related to the maturity of the cell preparations employed. Each of our experiments began with a unique population of theca cells, due to variability in the stage of sexual development of the ovaries of the pigs provided by the abattoir on any given day, so that although similar trends were observed in replicate experiments, the absolute values of steroid production varied among experiments. Because our experiments used ovarian tissue obtained from an abattoir, we could not induce follicular maturation experimentally with exogenous hormones as has been done in studies using the gonadotropin-primed rat model; therefore we could not be sure of the amount of gonadotropin to which our cells had been exposed in vivo.

In our studies, as in previous studies using other cell types, higher concentrations of insulin than those of Sm-C were required to elicit responses. The concentrations of insulin and Sm-C in porcine serum have been reported to be 0.38 ± 0.02 ng/ml and 1.26 ± 0.51 μg/ml, respectively, whereas porcine follicular fluid has been found to contain 0.48 ± 0.03 ng/ml insulin and 2.45 ± 0.39 μg/ml somatomedin C (Hammond et al., 1983). The follicular fluid insulin levels are generally thought to be too low to activate the high-affinity insulin receptor (Rein and Schomberg, 1982; Otani et al., 1985). In our studies, insulin effects were seen at higher than physiological concentrations, whereas the effects of Sm-C were seen in the normal physiological range. These results suggest that Sm-C rather than insulin may have a physiological role. Many cell types have been shown to possess separate receptors for Sm-C and insulin. Each peptide binds with high affinity to its own receptor and with lower affinity to the receptors for the related peptides. This occurs because of the

structural homologies between the receptors (Czech et al., 1983) for these molecules and the closely related structures of the peptides themselves (Zapf et al., 1981). Although the nature of the receptor in our studies remains to be determined, it is possible that our results were due to the interaction of high levels of insulin with the somatomedin receptor. To fully investigate whether insulin is acting through its own receptor or the receptor for a related insulin-like growth factor, characterization of these receptors in theca cells is necessary. To our knowledge, there have been no studies on porcine theca cell insulin or insulin-like growth factor receptors. The presence of insulin and/or insulin-like growth factor receptors have been characterized in rat theca-interstitial cells (Hernandez et al., 1988), porcine granulosa cells (Rein and Schomberg, 1982; Baranao and Hammond, 1984; Otani et al., 1985; Veldhuis et al., 1985b), human granulosa cells (Poretsky et al., 1985), and in the stroma and theca of human ovaries (Poretsky et al., 1985). Further caution is necessary in the interpretation of these results because insulin is rapidly inactivated in culture media (Mather and Sato, 1979), and it is possible that over the 48-h culture period the theca cells may have been exposed to significantly less bioactive insulin than was initially added to the cultures.

Our studies on the mechanism of action of insulin have shown that this hormone increases the accumulation of extracellular cAMP in addition to increasing steroid biosynthesis in both basal and LH-stimulated theca cell cultures. Our findings also show that insulin can interact with other cAMP-dependent agonists as indicated by the ability of insulin to enhance forskolin- and PGE_2-stimulated accumulation of extracellular cAMP and androstenedione. These findings suggest that the action of insulin is, at least in part, dependent on its ability to enhance the generation of cAMP under stimulation by adenylate cyclase activators. The insulin-induced increase in cAMP accumulation could be due to enhanced cAMP generation of decreased cAMP breakdown; however, the insulin effect in the presence of the phosphodiesterase inhibitor, MIX, favors the former interpretation. These findings are similar to results showing that Sm-C enhances cAMP accumulation by FSH-stimulated rat granulosa cells (Adashi et al., 1986).

It is clear from our studies of the site(s) in the steroidogenic pathway where insulin exerts its stimulatory effects that insulin causes a general increase in theca cell steroidogenesis. The data suggest that this involves an increase in the activity of both the cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase enzymes. These findings are consistent with the mechanism of action reported for insulin in the stimulation of granulosa cell progesterone production (Veldhuis et al., 1985a,b). Insulin increases granulosa cell progesterone production and causes a similar increase in the cellular cytochrome P-450 content associated with the cholesterol side-chain cleavage enzyme (Veldhuis et al., 1984).

Other studies have indicated that insulin has no significant effects on cell number, DNA content, plating efficiency, or cell viability in rat (Adashi et al., 1984) and porcine (Veldhuis et al., 1983) granulosa cells or in cultured rat theca-interstitial cells (Hernandez et al., 1988), suggesting that the stimulatory actions of insulin are not due to an enhancement of cellular replication or survival. The possibility that the increase in cAMP and steroidogenesis is due to an increase in theca cell number or survival has not been investigated in these studies; however, in the time course study (Fig. 3), it would appear that theca cell cultures containing LH and insulin would continue to produce androstenedione beyond the 48-h point, at a time when androstenedione production in cultures containing LH alone has plateaued. Although we have described two mechanisms that may contribute to the action of insulin, we cannot exclude the possibility that insulin may be acting through a generalized enhancement of the theca cells' metabolic capacity. In addition, insulin may increase LH receptor binding capacity and/or affinity, since studies on cultured porcine granulosa cells suggest that insulin is required for FSH-stimulated induction of LH receptors (May and Schomberg, 1981). Therefore, insulin may also be required for LH receptor induction in theca cells. Granulosa cells have been shown to be sites of insulin-like growth factor production, suggesting that they act in an autocrine manner to enhance FSH-stimulated responses in granulosa cells (Hammond et al., 1984). The possibility that these growth factors are produced locally in the theca, as they are in the granulosa cell, is an interesting possibility that has not yet been investigated.

From our data, it is clear that insulin and Sm-C can act directly on ovarian theca cells to increase androgen biosynthesis. The physiological significance of this finding is unclear, given that the concentration of insulin employed in these studies was higher than physiological levels. The data may be important physiologi-

cally in that insulin, or the related insulin-like growth factors, may play a role in follicular development since the androgens of theca cell origin play a role in follicular atresia and act as substrates for estrogen biosynthesis.

ACKNOWLEDGMENTS

We thank A. Rouleau and J. Rutledge for excellent technical assistance, and B. McDougall and N. Wilson for assistance in the preparation of this manuscript.

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