ABSTRACT  We investigated whether insulin plays a role in the proliferation and androgen production of chick testis cells. Testes from 18-d-old chick embryos or newly hatched chickens were dissociated and precultured in the presence of fetal bovine serum (FBS) for 24 h. After this period, testis cells from 18-d-old chick embryos were cultured in serum-free medium for 1 h with 0, 10, 50, or 100 µg/mL of insulin and were then exposed to human chorionic gonadotropin (hCG) for 3 h. In addition, some cells were incubated for 18 h with only insulin or insulin plus hCG. Androgens were measured by radioimmunoassay in the spent media. To study the influence of insulin on testis cell proliferation, cells were exposed to insulin for 18 h. A pulse of 3H-thymidine was added thereafter. We found that 18-d-old embryonic testis cells responded to hCG, increasing androgen production. Incubation with insulin for 1 h did not affect basal androgen production but modified the subsequent response to hCG. The addition of insulin plus hCG for 18 h resulted in important downregulation of the hCG effect. In addition, insulin significantly increased the proliferation of embryonic testis cells. The cells from testes of newly hatched chickens were precultured as described for embryonic cells and then exposed to insulin for 1 h in a serum-free medium. This treatment significantly increased the basal androgen production. Insulin also significantly enhanced the response to hCG of the testis cells from newly hatched chickens. These results strongly suggest that insulin has a role in the activity and in the proliferation of cultured testis cells throughout the perinatal period.

(Key words: testis cells, insulin, chicken, chick embryo, development)

INTRODUCTION

Maturation of the hypothalamic-pituitary-gonadal axis of the chick embryo is achieved by Day 13 of incubation (Woods et al., 1975, 1989; Woods, 1987). However, it has been demonstrated that very early in embryonic development, the chick is capable of synthesizing a variety of steroid hormones (Haffen and Cedard, 1968; Guichard et al., 1973). The testes of a 2-d-old chick embryo synthesizes testosterone in vitro (Connell et al., 1966). By using a cellular suspension incubated in vitro for 3 h, it has been demonstrated that testosterone production stimulated by human chorionic gonadotropin (hCG) remains high during the first week posthatching, but subsequently a rapid decline occurred, and the cells became refractory to such stimulation (Pedernera et al., 1987).

The roles of follicle-stimulating hormone, luteinizing hormone (LH), and hCG in the maintenance of testosterone production by the newborn chick testes in culture, as well as the influence of these hormones in response to gonadotropin stimulation has been reported previously by our group (Castro and Romano, 1994). We found that gonadotropins enhanced the production of androgens in a time-and-dose-dependent manner, suggesting that these hormones modulate steroidogenic activity of the neonatal population of chick testes (Castro and Romano, 1994).

Insulin and insulin-like growth factors are well recognized as having mitogenic activities in a variety of cell types (Straus, 1981). Insulin-like growth factor (IGF)-I is involved in the regulation of mammalian Leydig cells (Gelber et al., 1992; Saez, 1994; Lin, 1996). The direct effects of insulin on basal and hCG-stimulated accumulation of testosterone in primary cultures of rat testicular cells from adult hypophysectomized rats were reported by Adashi et al. (1982). These authors have demonstrated that insulin has no effect on basal testosterone production but increases the response to hCG in a time-and-dose-dependent way.

Synthesis of insulin in the chick embryo has been detected even before pancreatic beta cells were recognized (de Pablo et al., 1982). The hormone is also present in egg
constituents before fertilization (de Pablo et al., 1982). Insulin antibodies retard, and insulin accelerates, growth and differentiation in early chick embryos (de Pablo et al., 1985). Insulin receptors have been described in the brain of the chick embryo and many other tissues of the chick (Bassas et al., 1985, 1987; de Pablo and de la Rosa, 1995; Huhtaniemi and Toppuri, 1995). Furthermore, the development of the nervous system is influenced by insulin and IGF (de Pablo and de la Rosa, 1995). It was interesting to determine whether a hormone that is already present in the chick embryo, and affects its growth and differentiation, would have any influence on chick testes. To our knowledge there are no data on the possible role of this hormone in the chick testes function and development. In the present paper we studied the effect of insulin on the function and proliferation of chick testes. For this purpose we used the testes of embryonic and newly hatched chickens. Data presented here strongly suggest that insulin is an important hormone for the development and androgen production of chick testes.

MATERIAL AND METHODS

Cell Culture

Whole cell cultures from the testes of newly hatched (1-d-old) chicken or 18-d-old chick embryos (Rhode Island Red chickens obtained from a local hatchery) were prepared as described previously (Castro and Romano, 1994). Day-old chickens were killed by decapitation after ether anesthesia, and chick embryos were aseptically obtained from the egg. Testes were removed and placed in calcium and magnesium-free Hank’s solution and then incubated at 37°C in a 0.25% trypsin solution for 15 min. After this period, the albuginea was dissected out, and the parenchyma was cut into small pieces and further incubated in trypsin for 15 min. The tissue was then washed in Hank’s solution, placed in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and mechanically disrupted with a Pasteur pipette. Cell viability was tested by trypan blue exclusion; and more than 90% cells were viable (Ruth Bird and Forrester, 1981). Aliquots of cellular suspensions, corresponding to approximately two 1-d-old chick testes (5 × 10^6 cells/mL) were placed in each 35-mm Petri culture dish containing 2 mL of DMEM supplemented with 10% FBS and antibiotics (Penicillin 10,000 U/mL and streptomycin 10,000 mg/mL). Cells from chick embryo testes were seeded in 24-well cell culture clusters (approximately 1.5 testes/well, 4 × 10^6 cells/mL, more than 90% cells were viable by trypan blue exclusion) in the same media. The cells were incubated for 24 h at 37°C in a 5% CO2-95% air mixture.

Effect of Insulin on Chick Embryo Testes Cells

Androgen Production. After the preculture period described above, cells were washed, and the medium was changed for another containing DMEM plus 0.1% BSA and different doses of insulin (10, 50, and 100 µg/mL), and cells incubated for 1 h at 37°C. Control cells were incubated in the absence of insulin. After this period, media were discarded, and cells were further incubated for 3 h in the presence or absence (control) of 2 IU/mL hCG. At the end of this period, culture media were obtained, centrifuged, and ether-extracted as described previously (Castro and Romano, 1994). Released androgens were measured by RIA in the ether-extracted culture media as described later in this paper.

In some experiments, after the preculture period, cells were incubated for 18 h in the presence of insulin only or insulin plus hCG (2 IU/mL) in DMEM plus 0.1% BSA (control cells did not receive the hormones). After this period, media were recovered and ether-extracted. The production of androgens was measured by RIA.

Cell Proliferation. To investigate the effect of insulin on the proliferation of whole cultured testes cells, we dissected the testes from 18-d-old embryos, and a cell suspension was prepared as described above. Cells were precultured for 24 h in DMEM plus 10% FBS. After this period, the cultures were washed three times with DMEM and incubated for 18 h in DMEM plus 0.1% BSA in the presence or absence of different concentrations of insulin (10, 50, or 100 µg/mL). A pulse of 3H-Thymidine (2 Ci/m mole, 74 GBq/m mole, 1 mCi, 74 MBq) was then added, and the cells cultured for two additional hours.

At the end of this period cells were washed three times with PBS plus an excess of nonradioactive thymidine. The cultures were then exposed to 1 mL of 0.25% trypsin well for 15 min, scraped from the dishes, and placed in scintillation vials. Radioactivity was measured in a β-scintillation counter.

Effect of Insulin on the Testes of 1-D-Old Chickens

After the 24 h preculture period, media were changed to DMEM plus 0.1% BSA, and cells were cultured in the presence or absence (control) of different concentrations of insulin (6.2, 12.5, 25, 50, or 100 µg/mL) for 1 h. At the end of this period, media were extracted with ether, and the androgen production was measured as described for embryonic testes cells.

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To evaluate the effect of insulin on the hCG-stimulated production of androgens, cells were precultured in DMEM plus FBS for 24 h. Media were discarded, and cells were thereafter incubated for 1 h in DMEM plus 0.1% BSA and insulin (6.2, 12.5, 25, 50, or 100 µg/mL). Control cells did not receive the hormone. After this period, media were discarded and replaced by DMEM plus 0.1% BSA with or without (control) hCG (2 IU/mL), and the cells were further incubated for 3 h. Androgens were measured in the ether extract of the culture media.

**RIA**

Androgens present in the culture media were extracted twice with diethylether and measured by RIA (Adashi et al., 1982). The antibody that we used (antitestosterone) cross-reacted 18.75% with 5α-hydrotestosterone and 3% with 5α-androstane-α-17β-diol. 3H-Testosterone was used as the tracer (specific activity 98.8 Ci/Nm), and dextran-coated charcoal was used to separate the unbound tracer. The intra and interassay coefficients of variation were 5 and 11%, respectively.

**Statistical Analysis.** Experiments were replicated at least three times with three aliquots of cells each time (see legends of figures for details). Differences between treatments were tested using a computer program (GraphPad InStat) for ANOVA followed by Dunnet’s test.

### RESULTS

**Androgen Production by Testes of 18-d-old Chick Embryos**

The embryonic testes cells produced androgens in vitro and increased their secretion eightfold when hCG was added to the culture media (Figure 1). When the cells were preincubated for 1 h in the presence of insulin, the subsequent basal production of androgens was not modified. However preincubation with 10 µg insulin/mL modified the response of testes cells to hCG, inducing a small but significant increase in androgen production (Figure 1). Preincubation of cells with 100 µg insulin/mL caused significant inhibition of hCG-dependent androgen production.

At 18 h after the time the cells were exposed to insulin, there was a slight enhancement in basal androgen production (Figure 2). This figure also shows the effect of the incubation of testes cells for 18 h in the presence of insulin and hCG. Again, control cells (no insulin added to the culture media) increased the production of androgens significantly when hCG was present in the culture media. But, interestingly, the simultaneous presence of insulin and hCG for 18 h in the media significantly downregulated the effect of hCG in the three doses used in this study (Figure 2).

**Effect of Insulin on the 3H-Thymidine Incorporation to Embryonic Testes Cells**

Figure 3 shows the effect of the treatment with three different doses of insulin on the incorporation of 3H-Thymidine into cultured testes cells from 18-d-old embryos. The three doses of hormone significantly increased the uptake of 3H-thymidine by the testes cells. These results strongly suggest that insulin enhances the proliferation of embryonic cells.

**Androgen Production by the Testes of Newly Hatched Chickens**

As shown in Figure 4, the addition of increasing doses of bovine insulin for 1 h resulted in a significant increment of androgens in the culture media. The response of testes cells to insulin was dose-related. We did not observe a plateau even with the highest doses of insulin used in these experiments.

Figure 5 shows the response of testis cells after 3 h of incubation in the presence of hCG. Cells were previously
Effect of 18-h exposure to insulin only or insulin plus human chorionic gonadotropin (hCG) on testosterone production by embryonic testes cells. Testes cells from 18-d-old embryos were cultured for 24 h in Dulbecco’s Modified Eagle Medium (DMEM) plus 10% fetal bovine serum. Media were then discarded and replaced by DMEM-BSA in the absence (control cells) or presence of different concentrations of insulin (open circles). Another group of cells received only hCG (2 IU/mL) or the same dose of hCG plus different concentrations of insulin (filled circles). After 18 h of culture, testosterone production was measured in the media. Data are expressed as the mean ± SD of three experiments made in triplicate. *P < 0.05; **P < 0.01.

DISCUSSION

With a cell culture system we have investigated the effect of insulin on chick testes throughout the perinatal period. At this time of embryonic development, the hypothalamus-pituitary-gonadal axis is being established (Woods et al., 1975, 1989; Woods, 1987) and secretion of androgens is critical for brain development. This study reports for the first time the effect of insulin on the production of androgens and on cell proliferation in the testes of chicks.

We have previously demonstrated that testes cells from newly hatched chickens respond to gonadotropins by significantly increasing the production of androgens (Castro and Romano, 1994). By using a similar culture system, we now show that after 24 h of culture, testes cells from 18-d-old embryos also significantly increased the production of androgens in the presence of hCG. Interestingly, exposed to different concentrations of insulin for 1 h. After this period, media were discarded and replaced by another containing hCG. As can be observed in Figure 5 pretreatment with insulin clearly increased the response to a 3-h stimulus with hCG. The effect of insulin was again dose-related. It was also evident that 100 µg/mL downregulated the response to hCG.

FIGURE 2. Effect of 18-h exposure to insulin only or insulin plus human chorionic gonadotropin (hCG) on testosterone production by embryonic testes cells.

FIGURE 3. 3H-Thymidine incorporation in testes cells from 18-d-old chick embryos. The cells were cultured for 24 h in Dulbecco’s Modified Eagle Medium (DMEM) plus 10% fetal bovine serum. Media were then replaced by DMEM-BSA and insulin plus a pulse of 3H-thymidine was added. Cells were further incubated for 20 h. After this period they were washed and detached, and radioactivity was counted. Data are expressed as the mean ± SD of three experiments made in triplicate. *P < 0.01.

FIGURE 4. Effect of insulin on testosterone production by testes cells from a newly hatched chicken. The cells were cultured for 24 h in Dulbecco’s Modified Eagle Medium (DMEM) plus 10% fetal bovine serum. Media were then discarded and replaced by DMEM-BSA, and cells were further incubated for 1 h in the absence (control) or presence of different concentrations of insulin. At the end of this period, testosterone was determined in the media. Data are expressed as the mean ± SD of a representative experiment made in triplicate.
are expressed as the mean ± SD of a representative experiment made in triplicate.

FIGURE 5. Human chorionic gonadotropin-insulin interaction. Newly hatched chicken cells were cultured for 24 h in Dulbecco’s Modified Eagle Medium (DMEM) plus fetal bovine serum. Media were then discarded and replaced by DMEM-BSA, and cells were incubated for 1 h in the absence (control) or presence of different concentrations of insulin. After this period, media were replaced with DMEM-BSA plus human chorionic gonadotropin (2 IU/mL), and cells were further incubated for 3 h. Testosterone was measured in the extracted media. Data are expressed as the mean ± SD of a representative experiment made in triplicate.

embryonic cells did not significantly change basal androgen production when they were previously exposed to insulin, but those cells from newly hatched chicks did. These findings show that the testes of the chick can be influenced by insulin and that the stage of maturity affects the response of the cells to the hormone. Also, testes cells from 16.5-d-old, but not 20.5-d-old, rat fetuses increased production of testosterone when exposed to insulin for 3 d (Roulier-Fabre et al., 1998). Some authors have not found an effect of insulin on basal testosterone production when adult testes cells of the rat where used (Adashi et al., 1982; de Mellow et al., 1987). On the other hand, working with a purified Leydig cell preparation from adult rats (Lin et al., 1986) produced a mild stimulatory effect of insulin on basal testosterone production after 3 h of incubation with the hormone.

Data presented in this paper show that the stimulatory effect of insulin on basal androgen production by testes cells from the newly hatched chicken can be observed after only 1 h of exposure to insulin. The short time required for the effect of insulin could be explained by rapid actions of the hormone, such as stimulation of hexose, regulation of ion and amino acid transport, and modifications of the activities of rate-limiting enzymes that result in the activation of the enzymes involved in steroidogenesis (Rosen, 1987; Cheatham and Kahn, 1995; Saez, 1994; Lin, 1996).

Although we were not able to detect an important effect of insulin in the basal androgen production by embryonic cells, an interaction with hCG was found. The mild stimulatory effect on hCG-dependent androgen production found when embryonic cells were exposed to 10 µg insulin/mL was clearly improved when the cells were obtained from newly hatched chickens. A 1-h exposure of cells to insulin obtained from newly hatched chickens was sufficient to significantly enhance their response to hCG. Thereafter, the effect observed was not due to cell proliferation but was the result of an interaction of insulin with hCG. Again, this interaction was more evident in the newly hatched chicken, indicating that sensitivity to insulin is modulated by the developmental stage of the cells. Charreau et al. (1978) demonstrated, in vivo, the involvement of the hCG receptors on the effect of insulin on rat testes. However, as 1 h was sufficient to enhance the response of the cells from newly hatched chickens, it is probable that changes other than increased numbers of hCG receptors could be responsible of the effect of insulin on the gonadotropin-dependent production of androgens. Most probably, insulin may interact with hCG to stimulate the activity or expression of steroidogenic enzymes. Insulin and IGF-I have been shown to modulate the activity and expression of key steroidogenic enzymes in the placenta, adrenal, and ovary. Similarly, IGF-I modulates steroidogenic enzymes in rat testes (for reviews see Lin, 1996; Saez, 1994).

Interestingly, we found that in the newly hatched, as well as in the embryonic, chick testes a dose of 100 µg insulin/mL resulted in downregulation. Downregulation of hCG-stimulated androgen production was also observed when cells from newly hatched chickens were cultured in the presence of FBS for 24 h and further exposed to hCG (Castro and Romano, 1994). Interaction of insulin and hCG at the level of the hCG receptor, or on the production of cAMP, may explain the downregulation observed in embryonic and newly hatched testes cells. Regarding this point, it has been shown that IGF-I potentiates LH/hCG-supported cAMP production (for review see Saez, 1994), and this second messenger has direct, as well as indirect, roles in LH/hCG receptors downregulation (Dufau, 1988). Furthermore, high concentrations of cAMP decrease the transcription of the LH/hCG receptor gene (Nelson and Ascoli, 1992).

By using a whole-cell fetal testes culture system, this study reports for the first time a stimulatory effect of insulin on the proliferation of chick embryo testes cells. Long-term promoting effects of insulin on DNA synthesis, as well as on cell proliferation, have been found in several tissues (Cheatham and Kahn, 1995). The use of the 3H-thymidine incorporation technique in a whole-cell testis culture did not allow us to determine which cells types were involved in the insulin effect. However, preliminary results from our group in the same model have shown that 3β-hydroxysteroid dehydrogenase-positive cells, most probably Leydig cells, increase under the influence of insulin (unpublished results).

Thus, our present results demonstrate that insulin influences the proliferation and function of cultured chick perinatal testes cells. The mechanism mediating the in-
fluence of insulin on these cells needs further investigation. It is important to point out that we are using a whole testicular cell culture in which not only direct, but also paracrine, actions mediated by insulin might have occurred. Thus, the effect of insulin observed by us may be the result of not only a direct effect of insulin on Leydig cells but also of the effect of the hormone on Sertoli and peritubular cells. In vitro studies have shown that IGF-I, fibroblast growth factors, and tumor necrosis factor can be released into media by Sertoli, peritubular, and even Leydig cells (Saez, 1994). In summary, the results provided in this paper show that insulin modulates cell proliferation and steroidogenesis in cultured perinatal chick testes cells.

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