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Growth Factor Requirements for DNA Synthesis by Leydig Cells from the Immature Rat[1]

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ABSTRACT

Puberty in the male is dependent upon the elevated production of testosterone by the Leydig cells. LH affects this increase in testosterone output by increasing the total number of Leydig cells in the testis and by stimulating the steroidogenic pathway in these cells. Since Leydig cell proliferation is a prerequisite for the onset of puberty, we have examined the ability of LH and growth factors known to be present in the testis to promote DNA synthesis. Leydig cells were isolated from 21-day-old rats, cultured in serum-free medium for 48 h to become quiescent, and then treated with LH and growth factors for 18 h. [^{3}H]Thymidine incorporation into DNA was assessed over the subsequent 4-h incubation period. Cells in control cultures incorporated low levels of [^{3}H]thymidine into DNA and were stimulated after treatment with LH (100 ng/ml). Insulin/insulin-like growth factor-1 (IGF-1) and transforming growth factor-α (TGF-α), previously localized in Leydig cells by immunohistochemistry, also stimulated [^{3}H]thymidine incorporation into DNA. The responses of the Leydig cells to maximum levels of insulin and TGF-α were dependent on the cell density. Insulin and TGF-α alone and in combination increased the number of cells labeled with [^{3}H]thymidine, as assessed by autoradiography. TGF-β, known to be secreted by Sertoli cells, also stimulated DNA synthesis under basal conditions, but the maximum response was significantly lower than that achieved in the presence of TGF-α. Pretreatment of the cells with a low level of LH (2 ng/ml) for 48 h caused a marked enhancement of the response of the cells to the subsequent 18 h of treatment with insulin plus TGF-α and insulin plus TGF-β. In summary, whereas LH alone had little effect, it interacted synergistically with locally produced growth factors to promote DNA synthesis of immature Leydig cells. These interactions may determine the proliferative state of Leydig cells during development.

INTRODUCTION

Cell proliferation is regulated by endocrine, paracrine, and autocrine factors [1–2]. In the testes, the endocrine gonadotropins LH and FSH are required in vivo for the normal growth and differentiation of the Leydig and Sertoli cells and the initiation and progression of gametogenesis [3–5]. In hypophysectomized rats, spermatogenesis can be maintained qualitatively by the administration of testosterone [6–8]. Sartulli et al. [8] identified testosterone as a paracrine factor, secreted as a result of the action of LH on Leydig cells, that was essential for spermatogenesis to proceed.

During prepubertal development of the testis, the Leydig cells proliferate by an LH-dependent process to repopulate the intertubular space [9]. In hypophysectomized immature rats, the requirement for LH for Leydig cell proliferation cannot be substituted by testosterone [10], suggesting that LH stimulates Leydig cell proliferation directly or indirectly by regulating the production of growth-promoting factors that act in an autocrine manner. The growth factors transforming growth factor-α (TGF-α) and insulin-like growth factor-1 (IGF-1) have been localized in Leydig cells by immunohistochemical techniques [11–13]; however, their effects on Leydig cell proliferation have not been assessed. Other growth factors known to be secreted by Sertoli cells (e.g. transforming growth factor-β, TGF-β) [14, 15] may also diffuse into the intertubular space and influence the growth of the Leydig cells. Since growth factors are required for the progression through G_1 of the cell cycle leading to DNA synthesis and cell division [1], we have examined the effects of growth factors known to be present in the testis on DNA synthesis in immature Leydig cells.

MATERIAL AND METHODS

Materials

Ovine LH (NIDDK-oLH-17) and hCG (NICHD-NIAMDD, for iodination) were provided by the National Hormone and Pituitary Program NIDDK (Baltimore, MD). TGF-β (purified from porcine platelets) was purchased from R&D Systems Inc. (Minneapolis, MN) and was greater than 97% pure. Recombinant TGF-α was obtained from ICN Canada (Montreal, PQ, Canada), and insulin and IGF-1 from Gibco Chemical Company (Grand Island, NY). [^{3}H]Thymidine (81 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Collagenase (type 1) from Sigma Chemical Company (St. Louis, MO) was used for the preparation of Leydig cells.

Animals

Immature male Wistar Crl:(W1)BR rats were obtained from Charles River Canada (Montreal, PQ, Canada) at 19 days of age and were maintained with their mothers under conditions of controlled temperature (24°C) and light (12L:12D).

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Preparation of Leydig Cells

Rats were killed by cervical dislocation at 21 days of age and the testes were removed. The Leydig cell preparation was obtained as described previously [16, 17]. Briefly, the decapsulated testes were incubated for 15 min in culture medium containing 0.25 mg collagenase/ml. The seminiferous tubules were allowed to settle under unit gravity for 5 min, and the supernatant containing the suspension of interstitial cells was removed. The tubules were resuspended in medium, allowed to resettle, and the supernatant was collected. The pooled supernatants were centrifuged and the cell pellet was washed twice. The cells were plated into 24-multiwell tissue culture plates (Nunclon; Nunc Roskilde, Denmark) as 0.5-ml aliquots in culture medium. The medium consisted of Eagle's minimum essential medium (MEM) with Earle's salts and 0.1 mM of the following amino acid supplements: L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and glycine. The medium also contained 4 mM L-glutamine, 2.5 g/L $NaHCO_3$, 1.5 mM HEPES and antibiotics, 50 U/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml gentamicin (Gibco). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2: 95% air to maintain the medium at pH 7.4. After 1 h, the Leydig cells were well attached to the surface of the plate, whereas contaminating cells (Sertoli cells, germ cells) were loosely attached. At this time, the medium was discarded and the cells were washed three times with medium to remove loosely attached cells. At this stage, the cell culture contained more than 90% Leydig cells as assessed by staining for 3β-hydroxysteroid dehydrogenase. Fresh medium was added and the cells were cultured in serum-free medium for 48 h, in the presence or absence of a low level of oLH (2 ng/ml; NIDDK oLH-17).

DNA Synthesis by Leydig Cells

After 48 h in culture, the medium was removed and the cells were washed. Fresh medium was added and the cells were treated for 18 h with LH or growth factors. The medium was then replaced with 0.5 ml of fresh culture medium containing 0.5 μCi [^{3}H]thymidine and the cells were incubated for 4 h. The amount of [^{3}H]thymidine incorporated into DNA was determined as follows:

The medium was aspirated and the cells were washed with culture medium. PBS (0.5 ml) was added to each well and the cells were sonicated using a cell disruptor. Aliquots (200 μl) of the sonicate were filtered through Whatman DE-81 filter paper discs on a Millipore system (Millipore Corp., Bedford MA). The DNA remained bound to the DEAE-cellulose paper [18]. The filters were washed twice with 2 ml H_2O and counted in 5 ml CytoScint (ICN Biomedicals, Inc., Irvine, CA). The protein content of the sonicates was determined by the method of Lowry et al. [19].

Autoradiography

Leydig cells were cultured in 8-well culture chambers (Lab Tek Chamber Slides; Nunc) for 48 h, and treated with growth factors for the subsequent 18 h. [^{3}H]Thymidine (0.2 μCi/0.2 ml) was added to each chamber for 4 h, and the cells were fixed in 3% paraformaldehyde for 20 min. The slides were washed with PBS and stored in 70% ethanol. The slides were dipped in emulsion (Kodak NTB-2, Eastman Kodak, Rochester, NY), developed after 3 days, and stained with hematoxylin. At least 1 000 cells per chamber were counted using a light microscope adapted with Nomarsky optics. Cells were considered to be labeled when 10 or more silver grains were present above the nucleus. Leydig cells were identified by their spherical-to-oval nucleus, the distribution of heterochromatin, and the presence of lipid droplets [9]. Leydig cells are the only cells in the interstitium that contain lipid droplets [20]. An occasional labeled cell could not be identified as a Leydig cell by the above criteria, but the numbers were low (less than 1%).

Statistical Analysis

The results presented in Figures 1, 2, 4–6, and Table 1 are the means ± SD from three replicate cultures within each treatment group and are presented for a single representative experiment. Observations were confirmed in at least three independent experiments. Student's *t*-test and Duncan's multiple test were employed to assess the significance of differences between different treatments.

RESULTS

Leydig cells isolated from the testes of 21-day-old rats were maintained in culture in a serum-free medium for 48 h with no treatment to allow the cells to become quiescent. Since the proliferation of Leydig cells in vivo is an LH-dependent process, cells were treated with LH (100 ng/ml) for the subsequent 18-h culture period, and then incubated with [^{3}H]thymidine for 4 h. LH caused a small but reproducible increase in the level of [^{3}H]thymidine incorporated into Leydig cell DNA (Fig. 1; C vs. LH, solid bars, $p < 0.05$).

IGF-1 has been localized in the Leydig cells of the immature rat testis using immunocytochemical techniques [12, 13]. To determine if this growth factor influences the proliferation of the Leydig cells, its effects on DNA synthesis were assessed (Table 1). IGF-1 alone stimulated [^{3}H]thymidine incorporation into the DNA of cultured Leydig cells, 100 ng/ml producing a maximum response. The cells were unresponsive to 200 ng insulin/ml, whereas 1 μg insulin/ml stimulated [^{3}H]thymidine into DNA maximally (Table 1, Fig. 1). The requirement for high concentrations of insulin suggests that it may operate through the IGF-1 receptor with which it cross-reacts with a low affinity. Since insulin and IGF-1 exerted a similar effect on DNA synthesis, subsequent experiments were performed using insulin (1 μg/ml).

In sections through the 21-day-old rat testis, TGF-α has been localized by immunoperoxidase staining in Leydig cells

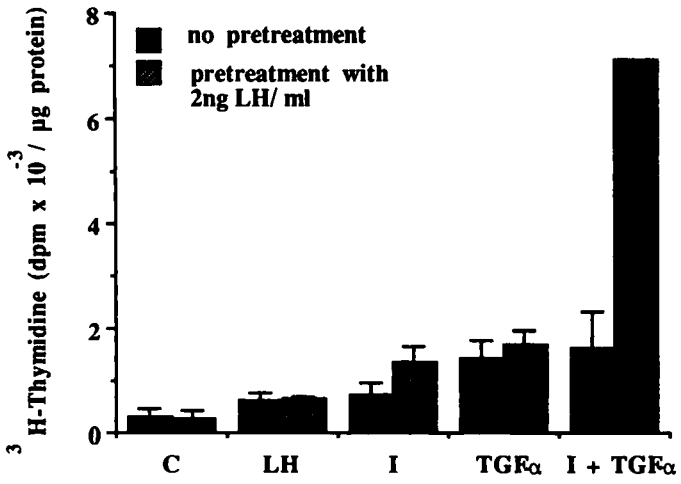

FIG. 1. [^{3}H]Thymidine incorporation into DNA by rat Leydig cells in culture treated with insulin and TGF-α. During the 48 h of culture from plating to the time of treatment, the Leydig cells were cultured in the absence (solid bars) or the presence (hatched bars) of LH (2 ng/ml). The cells were then treated for the subsequent 18 h with LH (100 ng/ml), insulin (I); (1 µg/ml) and/or TGF-α (10 ng/ml). Control cultures (C) were not treated during this 18-h period. [^{3}H]Thymidine incorporation per microgram of protein was determined after a 4-h incubation with 0.5 µCi [^{3}H]thymidine/0.5 ml culture medium. Data are means ± SD of three determinations.

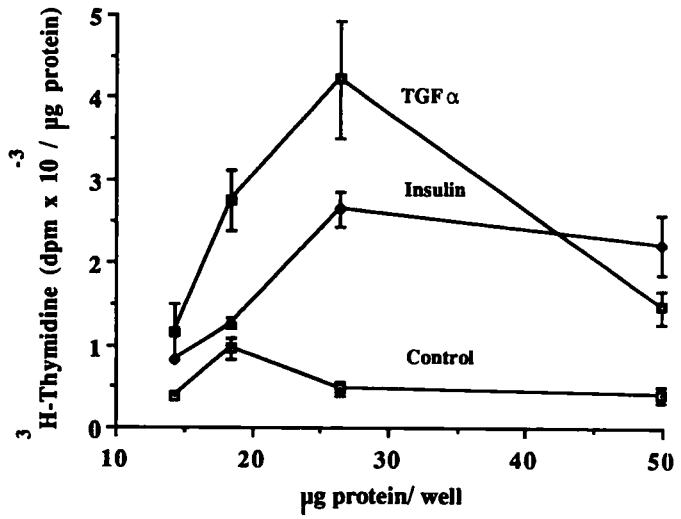

FIG. 2. [^{3}H]Thymidine incorporation into DNA by rat Leydig cells plated over a range of cell densities. After 48 h in culture with no treatment, cells were cultured for a further 18 h with no treatment (control) or treated with insulin (1 µg/ml) or TGF-α (10 ng/ml). The [^{3}H]thymidine incorporated per microgram of protein was plotted against the total cell protein present in the well at the end of the 4-h incubation with [^{3}H]thymidine.

[11]. The possibility that TGF-α may be involved in increasing the size of the Leydig cell population at this stage of development was supported by the ability of TGF-α to cause a 5-fold increase in DNA synthesis of cells in culture (Fig. 1). Treatment of the Leydig cells with insulin (1 µg/ml) plus TGF-α (10 ng/ml) did not significantly augment the response to TGF-α alone (Fig. 1; TGF-α vs. I + TGF-α, solid bars).

It has been shown previously that cultured Leydig cells lose steroidogenic response to LH. The responsiveness to LH stimulation can be maintained if the cells are cultured in the presence of a low concentration of LH (1–2 ng/ml) [21]. As shown in Figure 1, when cells were cultured from the time of plating in the presence of a low concentration of LH (2 ng/ml), no effect on the subsequent response to a higher concentration of LH (100 ng/ml) was found (LH solid bar vs. LH hatched bar). Pretreatment of cells with LH (2 ng/ml) had a synergistic effect on the response to insulin plus TGF-α (Fig. 1; I + TGF-α, solid bar vs. I + TGF-α, hatched bar).

As shown in Figure 2, the proliferative responses of the Leydig cells to either insulin or TGF-α were dependent on the cell densities. In the experiments presented in Figures 1, 3, 4, and 5, approximately 25 µg protein was present in each well at the end of the experiment.

The ability of insulin and TGF-α to re-initiate DNA synthesis in Leydig cells in culture was confirmed by autoradiography. As shown in the micrographs (Fig. 3) most of the cells that remained in culture for three days without treatment had become quiescent as judged by the low number (less than 1%) of cells that had incorporated [^{3}H]thymidine into the nucleus (Figs. 3 and 4). After treatment with insulin and TGF-α for 18 h before exposure to [^{3}H]thymidine, it was clear that many of the cells had proceeded through to the S-phase of the cell cycle, as assessed by evidence of [^{3}H]thymidine incorporation into the nucleus (Fig. 3). After treatment with insulin plus TGF-α, over 16% of the cells were labeled with [^{3}H]thymidine (Fig. 4).

TGF-β, known to be produced by rat Sertoli cells and peritubular cells, had a small but significant effect on DNA synthesis at 10 ng/ml but did not influence the response to insulin (Fig. 5). Pretreatment of cells with a low concentration of LH (2 ng/ml) significantly augmented the response to subsequent treatment with TGF-β (TGF-β solid bars vs. TGF-β hatched bars) and insulin plus TGF-β (I + TGF-β solid vs. I + TGF-β hatched bars). To compare the relative effectiveness of the respective growth factors, dose-

TABLE 1. Effects of insulin and IGF-1 on DNA synthesis by Leydig cells.[a]

	[^{3}H]Thymidine, dpm × 10^{-3}/µg protein
Control	0.35 ± 0.03
hCG (100 ng/ml)	0.56 ± 0.07
IGF-1: 50 ng/ml	0.69 ± 0.06[b]
100 ng/ml	1.02 ± 0.10[b]
200 ng/ml	1.08 ± 0.04[b]
Insulin: 200 ng/ml	0.59 ± 0.14
1 µg/ml	0.81 ± 0.05[b]
5 µg/ml	0.93 ± 0.20[b]

[a]Leydig cells were maintained in culture for 48 h with no pretreatment with LH. They were then treated with hCG alone or with insulin or IGF-1 alone for 18 h, then incubated for 4-h with [^{3}H]thymidine. The data are expressed as the means ± SD of the [^{3}H]thymidine incorporated into DNA/µg protein present at the end of the incubation.
[b]Significantly different from controls ($p < 0.05$).

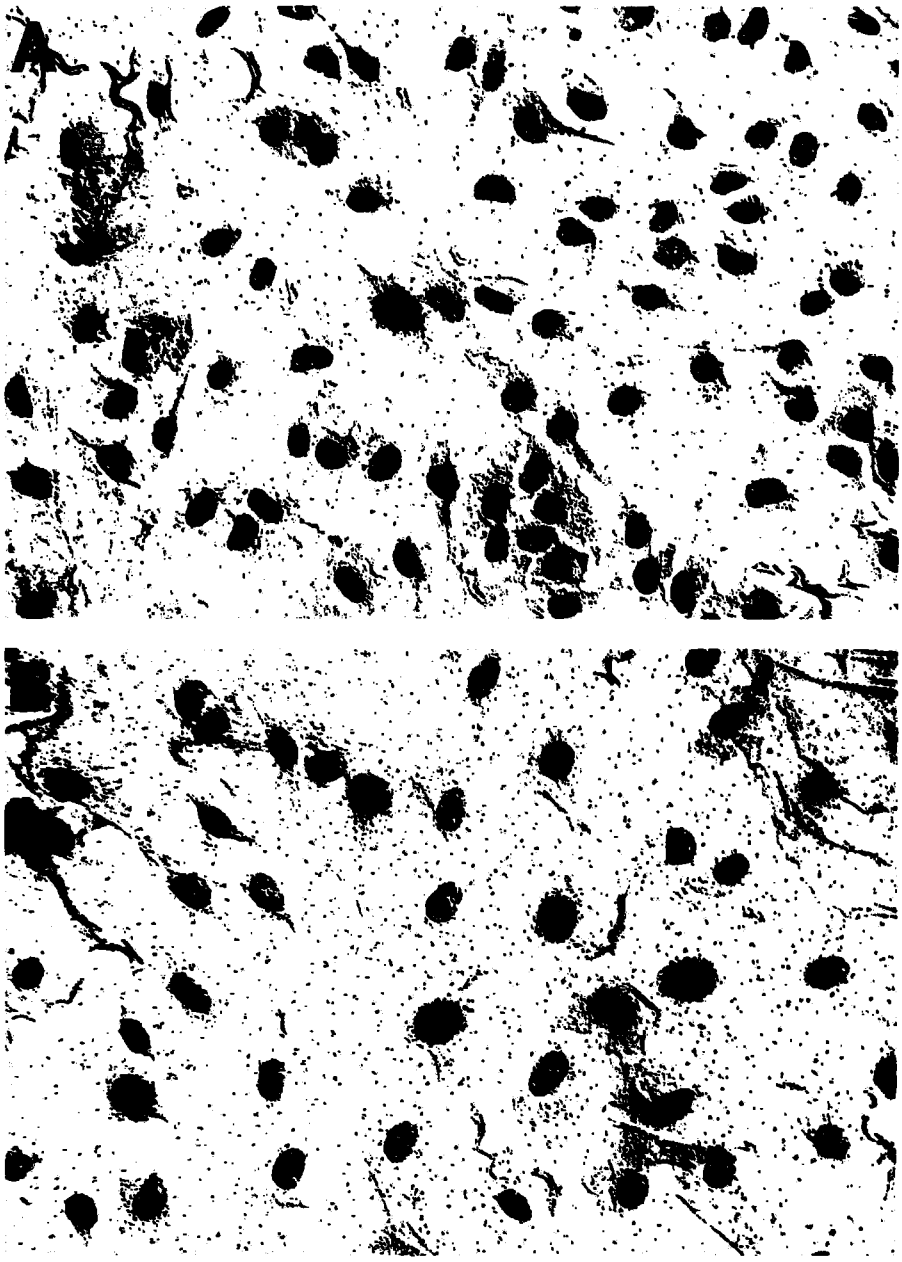

FIG. 3. Autoradiographs of Leydig cells after incubation with [^{3}H]thymidine. Cells were maintained in culture for 48 h without treatment. They were then treated with insulin plus TGF-α for the subsequent 18 h, followed by a 4-h incubation with [^{3}H]thymidine and autoradiography. A: untreated cells. B: cells treated with insulin and TGF-α. Total magnification ~ ×500.

response curves for TGF-α and TGF-β were generated. For both TGF-α and TGF-β, the maximum responses were achieved at 10 ng/ml. The [^{3}H]thymidine incorporation into DNA was stimulated to a greater extent by maximum concentrations of TGF-α than by TGF-β (Fig. 6).

DISCUSSION

In the testis of the adult mammal, the environment in which spermatogenesis proceeds is maintained by the actions of gonadotropins (LH and FSH) on the Leydig cells and Sertoli cells. To support germ cell development, the Leydig cell and Sertoli cell compartments expand by proliferation during discrete stages of prepubertal development. Once adequate populations of these cells have been generated, their secretion products maintain the highly synchronized cycles of germ cell proliferation and differentiation.

The expansion of the Leydig cell population occurs during the prepubertal period and is dependent upon the

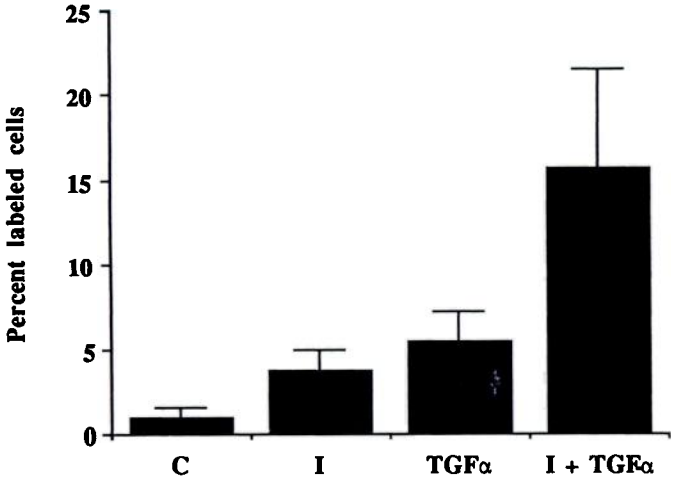

FIG. 4. Percent of labeled Leydig cells after autoradiography as described in legend to Figure 3. Cells were treated with insulin (I; 1 μg/ml) and TGF-α (10 ng/ml) either alone or in combination.

presence of LH. However, in the present study, treatment of Leydig cells from 21-day-old rats with a high concentration of LH in vitro stimulated DNA synthesis only to a limited extent. The latter finding raised the possibility that the actions of LH require the interaction with other factors that were not present under the prevailing culture conditions, or, that the actions of LH were mediated indirectly through the production of autocrine factors that were not generated in sufficient amounts in culture to effectively stimulate DNA synthesis. Since growth factors are required for the progression through the cell cycle, we have examined the growth factor requirements of Leydig cells that allow them to repopulate the intertubular space and determined if these growth factors interact with LH. The novel findings presented here show that IGF-1, TGF-α, and TGF-β are potential key growth factors for Leydig cell proliferation. Furthermore, interactions between LH, IGF-1, and TGF-α provide the optimum combination of intracellular signals for DNA synthesis.

We have recently demonstrated that immunoreactive TGF-α is present in Leydig cells at the onset of their proliferation phase in the 21-day-old rat. At this time, when mesenchymal cells differentiate into Leydig cells, TGF-α can be visualized in the majority of the Leydig cells. Previously, epidermal growth factor (EGF) has been shown to influence steroidogenesis in Leydig cells. Verhoeven and Cailleau [21] showed that EGF stimulates steroidogenesis in freshly isolated Leydig cells. TGF-α has sequence homology with EGF and binds with high affinity to the EGF receptor exerting similar biological responses [22, 23]. In the rat testis, TGF-α appears to be the important factor, since the gene for TGF-α is transcribed and not the gene for EGF [24]. The demonstration that TGF-α is a mitogen for Leydig cells identifies an additional physiological role for this growth factor in vivo. It is not clear if the TGF-α present in the Leydig cells is synthesized there or is taken up from the interstitial fluid. In other cells from endocrine tissues, such as pituitary cells and thecal cells, the immunoreactive cells are the source of TGF-α [25, 26].

Theoretically, the TGF-α present in Leydig cells can influence adjacent cells in two ways depending upon the subsequent processing of the peptide. The TGF-α gene transcribes a precursor molecule that contains a transmembrane domain that anchors the molecule in the plasma membrane of the cell in which it is synthesized. The native form of the TGF-α is present in the extracellular portion of the precursor molecule and can be cleaved and released into the extracellular environment [27]. Presumably this soluble peptide can then diffuse short-range from its site of synthesis to act as an autocrine or paracrine regulator of growth and differentiation. Another mode of action of TGF-α may be elicited as it remains anchored in the plasma membrane, by direct interaction of the extracellular domain of the TGF-α molecule with receptors on adjacent cells [28, 29].

Immunoreactive IGF-1 has also been localized in Leydig cells [12, 13]; however, the intensity of staining is greater in Sertoli and spermatogenic cells [13]. Leydig cells contain mRNA for IGF-1 [30] and secrete IGF-1 and IGF-1-binding proteins in culture [31]. With respect to the physiological role of IGF-1 production in Leydig cells, augmentation of hCG-induced testosterone production by rat Leydig cells has been demonstrated [32], and the present study identifies a role for IGF-1 in the stimulation of DNA synthesis. In addition to these autocrine actions of IGF-1 produced by Leydig cells, paracrine actions on other cells in the interstitium or in the seminiferous tubule may also occur. Conversely, the higher level of IGF-1 found in tubular cells may contribute to the pool of IGF-1 in the interstitial space and exert paracrine effects on Leydig cell functions.

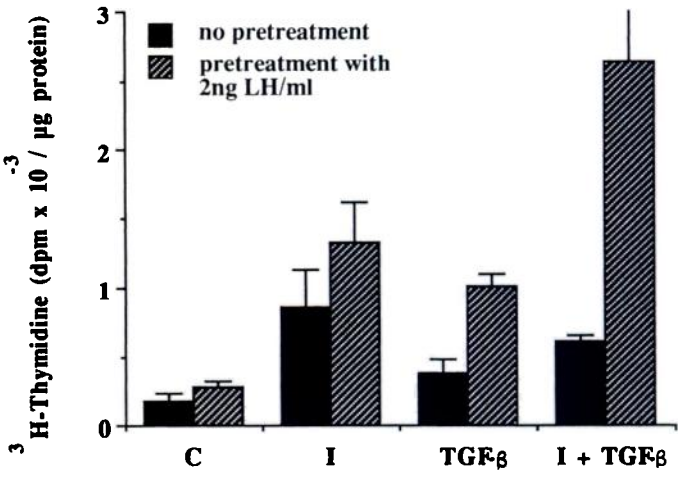

FIG. 5. [^{3}H]Thymidine incorporation into DNA by rat Leydig cells in culture treated with TGF-β. Cells were either untreated (solid bars) or pretreated with LH (2 ng/ml; hatched bars) as described in legend to Figure 1. The cells were then treated for the subsequent 18 h with LH (100 ng/ml), insulin (I; 1 μg/ml), and/or TGF-β (10 ng/ml). Control (C) were not treated during this 18-h period.

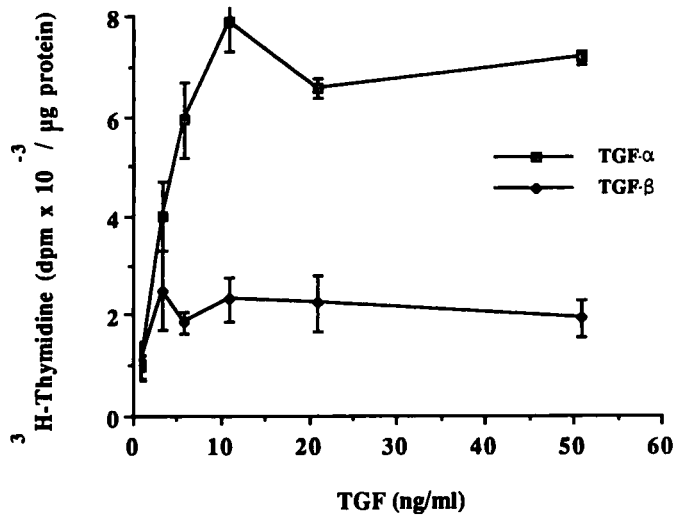

FIG. 6. [^{3}H]Thymidine incorporation into DNA by rat Leydig cells treated with a range of concentrations of TGF-α and TGF-β. After 48 h in culture with no treatment, the cells were cultured for a further 18 h with a range of concentrations of TGF-α and TGF-β. [^{3}H]Thymidine incorporation per microgram of protein was determined after a 4-h incubation with 0.5 µCi [^{3}H]thymidine/0.5 ml culture medium. Data are means ± SD (three determinations.)

The temporal sequence of the induction of gene expression for IGF-1 and TGF-α in the Leydig cell is not precisely known. Nevertheless, during the prepubertal period the production of either IGF-1 or TGF-α would allow the initiation of DNA synthesis in a proportion of the cells. As the second component is induced, interactions would result in a greater proportion of the cells entering the cell cycle. Also during the prepubertal stage, LH levels become elevated. From a physiological standpoint, therefore, it was interesting to find that pretreatment of the cells with LH resulted in a significant augmentation of the insulin plus TGF-α response.

The mechanisms by which LH, IGF-1, and TGF-α interact to allow Leydig cells to enter the cell cycle and initiate DNA synthesis is not known. Nevertheless, one can speculate that IGF-1 and TGF-α act as progression factors allowing the progression of the G_1 phase of the cell cycle [1].

LH may influence Leydig cell proliferation in a number of different ways. It may promote the differentiation of mesenchymal cells into Leydig cells, resulting in changes in gene expression for growth factors and growth factor receptors. Alternatively, by virtue of its ability to increase intracellular cAMP, LH may have a direct effect on the progression of the cell cycle. Several hormones, that influence differentiated functions of their target cells through cAMP as a second messenger also promote cell proliferation. For example, FSH (and cAMP) interact with TGF-β to stimulate aromatase activity and to promote DNA synthesis in rat granulosa cells [33], and TSH action through cAMP increases the synthesis of thyroid hormones and proliferation of thyrocytes [2]. In the Leydig cells, there is synergism between LH (mediated by cAMP) and factors (e.g. IGF-1, TGF-α) acting through cAMP-independent mechanisms to promote growth. A similar situation occurs in thyrocytes, where the mitogenic actions of TSH, mediated through cAMP, are dependent on insulin or IGF-1 and augmented by the presence of EGF [2].

TGF-β is secreted by Sertoli cells [14, 15] and may diffuse into the interstitial space to influence Leydig cell functions. As shown in Figure 5, TGF-β alone can stimulate [^{3}H]thymidine incorporation into DNA, and this response is enhanced after pretreatment of the cells with LH. Relative to TGF-α, the responses to a range of concentrations of TGF-β are small (Fig. 6), suggesting that the physiological functions of TGF-β at this stage of development are directed towards modulation of steroidogenesis [34] rather than promoting the growth of Leydig cells.

In summary, DNA synthesis by Leydig cells from immature rats depends upon the extracellular signals that they receive from hormones and growth-promoting factors. In the present study we have shown that LH alone is minimally effective in stimulating DNA synthesis in vitro and depends upon the presence of insulin/IGF-1 and TGF-α for maximal stimulation. These interactions between LH and growth factors acting in an autocrine manner may determine the proliferative state of the Leydig cells during development.

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