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Lutropin/Choriogonadotropin Stimulate the Proliferation of Primary Cultures of Rat Leydig Cells through a Pathway that Involves Activation of the Extracellularly Regulated Kinase 1/2 Cascade

Koji Shiraishi and Mario Ascoli

Department of Pharmacology, Carver College of Medicine, The University of Iowa, Iowa City, Iowa 52242

Primary cultures of progenitor and immature rat Leydig cells were established from the testes of 21- and 35-d-old rats, respectively. The cell population remained homogeneous after 4–6 d in culture as judged by staining for 3β-hydroxysteroid dehydrogenase, but the cells were unable to bind ^{125}I-human chorionic gonadotropin (hCG) or to respond to hCG with classical LH receptor (LHR)-mediated responses, including cAMP and inositol phosphate accumulation, steroid biosynthesis, or the phosphorylation of ERK1/2. Infection of primary cultures with recombinant adenovirus coding for β-galactosidase showed that approximately 65% of the cells are infected. Infection with adenovirus coding for the human LHR (hLHR) allowed for expression of the hLHR at a density of approximately 25,000 receptors per cell and allowed the cells to respond to hCG with increases in cAMP and inositol phosphate accumulation, steroid biosynthesis, and the phosphorylation of ERK1/2. Although progenitor and immature cells were able to respond to hCG with an increase in progesterone, only the immature cells responded with an increase in testosterone. In addition to these classical LHR-mediated responses, the primary cultures of progenitor or immature rat Leydig cells expressing the recombinant hLHR proliferated robustly when incubated with hCG, and this proliferative response was sensitive to an inhibitor of ERK1/2 phosphorylation. These studies establish a novel experimental paradigm that can be used to study the proliferative response of Leydig cells to LH/CG. We conclude that activation of the LHR-provoked Leydig cell proliferation requires activation of the ERK1/2 cascade. (*Endocrinology* 148: 3214–3225, 2007)

INJECTIONS OF LH/CHORIONIC gonadotropin (CG) were shown to stimulate the proliferation and differentiation of Leydig cells and to induce Leydig cell hyperplasia in rodents many years ago (1–3). The ability of the LH receptor (LHR) to stimulate the proliferation of Leydig cells and to behave as a potential Leydig cell oncogene was not generally recognized until recently, but when the phenotypes of boys harboring naturally occurring mutations of the human (h) LHR gene were described previously (for review, see Refs. 4–7). 46XY individuals harboring germ-line activating mutations of the hLHR have Leydig cell hyperplasia, whereas those harboring germ-line inactivating mutations of the hLHR have Leydig cell hypoplasia. In addition, the finding of a somatic activating mutation of the hLHR in Leydig cell adenomas of several unrelated boys with precocious puberty (8–10) suggests that the LHR could even be involved in the transformation of Leydig cells. The mitogenic and oncogenic potential of the LHR is also supported by other recent observations made in genetically modified mouse models (for review, see Refs. 11–13). For example, mice lacking GnRH as well as mice with targeted deletion of the LHR exhibit Leydig cell hypoplasia (14–16), whereas transgenic mice overexpressing hCG or LH develop Leydig cell adenomas (13, 17). LH administration can also induce the development of Leydig cell tumors in other transgenic mice models (18, 19), and wild-type mice with high levels of LH induced by administration of 5α-reductase inhibitors (20) display an increased incidence of gonadal tumors. Lastly, when ectopically expressed in the adrenal cortex, the LHR induces gonadotropin-dependent adrenocortical hyperplasia or adrenocortical tumors (21, 22).

These data lead us to postulate that the LHR activates signaling cascades that promote the proliferation and/or survival of Leydig cells.

There is a growing body of recent evidence from many different laboratories that implicate the LHR as a stimulant of mitogenic and/or survival signaling cascades such as the ERK1/2 pathway. The phosphorylation of ERK1/2 is increased by LH/CG in primary cultures of granulosa cells (23, 24), immortalized granulosa cell lines (25), MA-10 Leydig tumor cells (26, 27), and primary cultures of immature rat Leydig cells (28). Although this signaling pathway is emerging as an important regulator of steroidogenesis in Leydig (28), granulosa (25, 29), and Sertoli (30) cells, there are no reports examining its potential involvement as a mediator of the LHR-provoked proliferation of Leydig cells.

Methods to isolate homogenous populations rat Leydig cells of different stages of differentiation and to maintain

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Abbreviations: Ad, Adenovirus; βgal, β-galactosidase; CG, chorionic gonadotropin; CMF-HBSS, Hanks' balanced salt solution without Ca^{+2} or Mg^{+2}; DHT, dihydrotestosterone; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; h, human; 3β-HSD, 3β-hydroxysteroid dehydrogenase; rILC, immature rat Leydig cell; LHR, LH receptor; MEK, MAPK kinase; MOI, multiplicity of infection; PKA, protein kinase A; rPLC, progenitor rat Leydig cell; TBS, Tris-buffered saline.

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them in short-term primary culture have been established previously by several laboratories (28, 31–33). Although freshly isolated Leydig cells of different stages of differentiation express the LHR (32, 34, 35) and display a variety of acute and classical LHR-mediated responses such as cAMP accumulation, steroidogenesis, and ERK1/2 activation (28, 31–33), they are not very useful in the study of proliferation for at least two reasons. Freshly isolated rat Leydig cells loose viability, display a reduced rate of DNA replication, and become apoptotic during the first 48 h after isolation (36), and proliferation assays are best done using cells that have been allowed to become quiescent by serum deprivation (37, 38). Therefore, freshly isolated Leydig cells have to be incubated in serum-free medium for 24–48 h before testing the effects of hormones on proliferation (36, 38, 39).

Maintaining primary cultures of Leydig cells for several days would be more useful in the study of Leydig cell proliferation, but maintaining gonadotropin responsiveness over a period of days in culture is a more complicated process. Primary cultures or rat Leydig cells maintained for 1–3 d attached to Cytodex beads and using culture medium supplemented with low levels of partially purified LH alone or together with dihydrotestosterone (DHT) display LHR-mediated responses such as cAMP and steroidogenesis (32, 34, 35). It is not clear whether these conditions are also beneficial to cell proliferation, however. In fact, attempts to demonstrate an effect of LH/CG on DNA replication in short-term cultures of rat Leydig cells maintained with or without low levels of LH have not always been successful (38–41).

In this paper, we show that progenitor (rPLCs) or immature (rILCs) rat Leydig cells expressing the recombinant LHR can be maintained in primary culture for several days and that they proliferate in response to LHR activation. Using these primary cultures and inhibitors of the ERK1/2 cascade, we tested the hypothesis that this signaling pathway is a mediator of the LHR-provoked proliferation of Leydig cells.

Materials and Methods

Isolation, culture, and infection of Leydig cells

rPLCs and rILCs were isolated from testes of 21- and 35-d-old rats, by Percoll gradient centrifugation (28, 33). Decapsulated testes were incubated with type I collagenase (0.25 mg/ml) for 20 min at 37 C, and the digested tissue was passed through a 70-μm cell strainer. The filtrate was centrifuged at 250 $\times$ *g* for 8 min at room temperature, and the pellet was washed once by centrifugation with Hanks' balanced salt solution without Ca^{+2} or Mg^{+2} (CMF-HBSS) containing 1 mg/ml BSA (CMF-HBSS-BSA). To obtain purified Leydig cells, this crude cell suspension was loaded on top of a discontinuous gradient consisting of layers of 5 ml of 20% Percoll, 10 ml of 40% Percoll, 10 ml of 60% Percoll, and 3 ml of 90% Percoll (all made in CMF-HBSS) and subsequently centrifuged at 800 $\times$ *g* for 20 min at room temperature. The third band of cells from the top was collected, diluted with 2 vol of CMF-HBBS-BSA and centrifuged at 350 $\times$ *g* for 10 min at room temperature. The pellet was resuspended in CMF-HBSS-BSA, mixed with 90% Percoll (containing color beads of a density of 1.068 g/ml) to give a final Percoll concentration of 60%, and centrifuged at 20,000 $\times$ *g* for 30 min at 4 C. The Percoll fraction with a density lower than 1.068 g/ml was discarded, and the higher density fraction (containing purified Leydig cells) was diluted with 2 vol of CMF-HBSS-BSA and centrifuged at 350 $\times$ *g* for 10 min at room temperature. The purified Leydig cells were resuspended in culture medium (DMEM/F12) supplemented with 15 mM HEPES (pH 7.4), BSA (1 mg/ml), and gentamicin (100 μg/ml), centrifuged again, resuspended in the same medium (BSA-containing medium), and counted. Cell yields were approximately 0.3×10^6 and approximately 2×10^6/rat for the 21- and 35-d-old rats, respectively. These procedures were approved by the Institutional Animal Care and Use Committee for the University of Iowa.

Cells were plated in DMEM/F12 with 0.1% BSA in gelatin-coated 12-well plates at a density of 1×10^5 (progenitor) or 2×10^5 (immature) cells per well, in a total volume of 1.0 ml of medium. The cell culture plasticware was coated with gelatin as described previously (42). One day after plating, the culture medium was changed to DMEM/F12 supplemented with 15 mM HEPES (pH 7.4), 2% newborn calf serum, and 100 μg/ml gentamicin (serum-containing medium). Two days after plating, some wells were trypsinized (43) and counted, and the rest were washed twice with BSA-containing medium and incubated with the recombinant adenovirus coding for the hLHR (Ad-hLHR) or for β-galactosidase (Ad-βgal) used at 200 multiplicity of infection (MOI = number of viable viral particles per cell) for 2 h at 37 C in a total volume of 1 ml (44). At the time of infection (d 2), the number of attached progenitor or immature cells was approximately 0.5×10^5 cells per well. The infection solution was then aspirated and replaced with serum-containing medium to prevent additional infection. The following day (3 d after plating) the medium was changed again to BSA-containing medium, and all experiments were initiated 4 d after plating (2 d after infection). On d 4, there were approximately 1×10^5 cells per well for progenitor and immature cells, and the wells were approximately 40% confluent.

Immunocytochemistry

On d 4, the cells were fixed using 4% paraformaldehyde dissolved in 10 mM sodium phosphate, 150 mM NaCl (pH 7.4) (PBS) for 10 min at 4 C. After washing twice with 10 mM Tris, 150 mM NaCl (pH 7.4) [Tris-buffered saline (TBS)], the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. The cells were processed with the avidin-biotin complex method using the Vectastain ABC kit (Vector Laboratories, Burlington, CA) according to the instructions of the manufacturer. The fixed cells were incubated for 60 min at room temperature in a solution of 0.5% goat serum, 0.1% Triton X-100 in TBS. The cells were then incubated overnight at 4 C with a 1:1000 dilution of a rabbit antiserum to 3β-hydroxysteroid dehydrogenase (3β-HSD) or normal rabbit serum in 0.1% Triton X-100 in TBS. After washing twice with 0.1% Triton X-100 in TBS, the cells were treated with biotinylated antirabbit IgG (1:500 dilution) in TBS containing 3% BSA for 1 h at room temperature. This was followed by a 30 min incubation with 0.6% hydrogen peroxide in TBS. Then, the avidin-biotin complex reagent was applied for 1 h, and the immune complexes were revealed with 3,3′-diaminobenzidine chromogen (prepared according to the instructions of the manufacturer) for 10 min at room temperature. The reaction was stopped by adding 1 ml of water, and the cells were examined and photographed with a phase contrast microscope.

βgal staining

On d 4, the cells were washed twice with 10 mM sodium phosphate, 150 mM NaCl (pH 7.4) (PBS) and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 min at room temperature. The cells were then washed again with PBS and stained with a solution of 1 mg/ml βgal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$, all dissolved in PBS. After an overnight incubation at 37 C, the cells were washed twice with PBS. The percentage of infected cells was calculated by scoring 300 cells from each transfection for the presence of a blue precipitate in the cytosol.

Cell counting

On d 4, the medium was replaced and the cells were then incubated with or without hormones (in a total volume of 1 ml of BSA-containing medium). The cells were counted on d 5 and 6, but the medium and hormones were replaced on d 5 for the cells that were counted on d 6. The cells were trypsinized as described for MA-10 cells (43) and counted in a Beckman Coulter (Fullerton, CA) counter.

[^{3}H]Thymidine incorporation

The cells were incubated with or without hormones for 24 h as described for the cell counting experiments. [^{3}H-methyl]thymidine (2

μCi/ml) was added during the last 4 h of the incubation. The cells were washed twice with 1 ml of 20 mM HEPES, 150 mM NaCl (pH 7.4) and then incubated with 0.5 ml of cold 10% trichloroacetic acid for 30 min on ice. After aspiration, the cells were washed twice with 0.5 ml of the same solution. The cells were then dissolved in 500 μl of 0.5 N NaOH, mixed with 10 ml of BudgetSolve, neutralized with 125 μl of 2 N HCl, and counted in a liquid scintillation counter.

Thymidine incorporation in freshly isolated cells was measured in suspended cells incubated with [^{3}H-methyl]thymidine (2 μCi/ml) for 4 h. These cells were washed by centrifugation using the buffers described above. After precipitation of the cell pellets with trichloroacetic acid, the precipitates were solubilized and counted as described above.

Hormone binding, second-messenger, and steroid assays

All of these assays were also done on d 4. Binding assays were done during a 1-h incubation at room temperature with 100 ng/ml ^{125}I-hCG with or without an excess of nonradioactive hCG (to correct for nonspecific binding) as described previously (42). Steroids and cAMP were measured using enzymatic immune assays using commercially available kits (progesterone and testosterone; Cayman Chemicals, Ann Arbor, MI) or by RIA (cAMP) using reagents prepared in our own laboratory. For these assays, the cells were incubated with or without hCG (100 ng/ml) for 4 h in the presence of 1 mM isobutylmethylxanthine (to inhibit cAMP phosphodiesterases) as described previously (42). Inositol phosphate accumulation was measured in cells prelabeled with [^{3}H]myoinositol (42) and incubated with or without hCG (500 ng/ml) for 1 h in the presence of 20 mM LiCl (to inhibit the degradation of inositol phosphates). The concentrations of hCG used are the minimal concentration of hCG that elicit maximal responses in each of these assays (42).

Western blots

These methods have also been described (27, 42, 45). Primary antibodies to phospho-ERK1/2, total ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), P450^{scc} (Chemicon, Temecula, CA), and 3β-HSD (a generous gift from Dr. Anita Payne, Stanford University, Palo Alto, CA) were used at dilutions of 1:5000, 1:10,000, 1:1000, and 1:1000, respectively. Antimouse and antirabbit secondary antibodies were used at dilutions of 1:10,000 and 1:3000, respectively.

Statistical analysis

A two-tailed *t* test was used for comparing two groups (see Figs. 2B, 7A, and 9), ANOVA with Dunnett's *post hoc* test was used for multiple comparisons to a control group (see Figs. 5 and 6), and ANOVA with Bonferroni's *post hoc* test was used for multiple comparisons among groups (see Figs. 3, 4, 7B, and 8). These analyses were performed using the InStat Software package from Graphpad Software (San Diego, CA). In all cases, statistical significance was considered at $P < 0.05$.

Hormones and supplies

Purified hCG (CR-127) was purchased from Dr. A. Parlow of the National Hormone and Pituitary Agency (Torrance, CA). Purified recombinant hCG was kindly provided by Ares Serono (Randolph, MA). AG1478, AG43, PP2, PP3, GM6001, H89, PD98059, 8-CPT-cAMP, N^6-benzoyl-cAMP, and 8-CPT-2Me cAMP were from Calbiochem (San Diego, CA). U0126 was from Tocris (Ellisville, MO). Cell culture medium was obtained from Invitrogen (Carlsbad, CA), and cell culture plasticware was from Dow Corning (Corning, NY). [^{3}H-methyl]Thymidine (20 Ci/mmol) and [^{3}H]myoinositol (10 Ci/mmol) were from PerkinElmer (Boston, MA). All other chemicals were obtained from commonly used suppliers.

Results

Establishment and characterization of primary cultures of rat Leydig cells

We reasoned that establishing long-term primary cultures of Leydig cells that maintain gonadotropin responsiveness in the absence of gonadotropins would be a desirable goal to study the proliferative response of Leydig cells to LH/CG. To address these issues, we attempted to establish primary cultures of Leydig cells from 21- and 35-d-old rats that could be maintained for at least 6 d. Animals of these two ages were chosen because there is agreement that the testes of 21-d-old rats contain mostly rPLCs, whereas those of 35-d-old rats contain mostly rILCs and that these two populations of Leydig cells have the capacity to proliferate (46, 47). The 6-d culture period was arbitrarily chosen because it was considered long enough to examine the proliferative potential and responsiveness of the primary cultures.

Figure 1A shows that, after 4 d, our primary cultures contain mostly (or entirely) Leydig cells as judged by staining for 3β-HSD. As expected, the level of 3β-HSD is higher in immature than in the progenitor cells (34, 46, 47). The expression of a second Leydig cell marker (the cholesterol side chain cleavage enzyme P450^{scc}) can also be readily detected on Western blots as shown in Fig. 1B. The level of this enzyme was the same in both cell stages, however.

After 4 d, these primary cultures do not retain significant levels of endogenous LHR as judged by ^{125}I-hCG binding (see Fig. 2B), and their LHR-mediated responses are weak or absent (see Fig. 3). In an attempt to restore ^{125}I-hCG binding and hCG responsiveness, we infected the primary cultures with Ad-βgal or Ad-hLHR and tested for expression of the encoded proteins by enzymatic activity (βgal) or by ^{125}I-hCG binding (hLHR). The results presented in Fig. 2A show that, when the primary cultures are infected with Ad-βgal at an MOI of 200, about 65% of the Leydig cells stain for βgal activity. When infected with Ad-hLHR at an MOI of 200, the primary cultures bind approximately 2 ng ^{125}I-hCG/10^6 cells. Lowering the MOI to 20 resulted in little or no infection, and increasing it to 500 increased the percentage of infected cells (measured by staining for βgal activity) to approximately

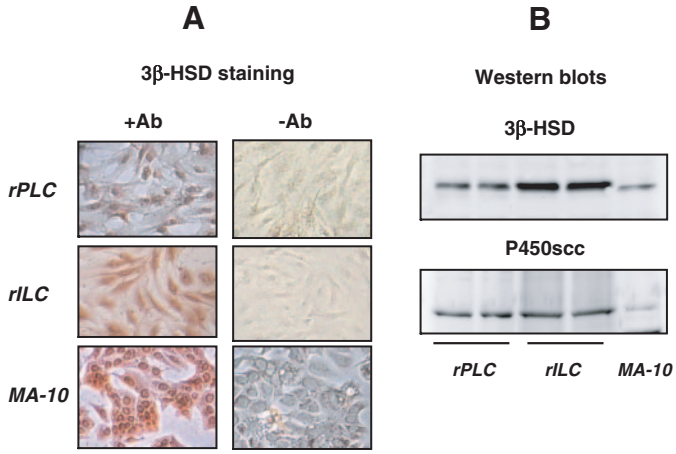

FIG. 1. Primary cultures of rPLCs and rILCs express markers of Leydig cell lineage. Rat Leydig cells were isolated from 21-d-old (rPLC) or 35-d-old (rILC) rats and maintained in primary culture as described in *Materials and Methods*. MA-10 cells were cultured as described previously (42, 43) and were used as positive controls. A, Four-day-old cultures were stained with (+Ab) or without (−Ab) an antibody for 3β-HSD. The results of a representative experiment are shown. B, Western blots of whole-cell lysates from two different 4-d-old cultures were probed with antibodies to 3β-HSD or P450^{scc} as shown. Only the relevant portions of the blots of a representative experiment are shown.

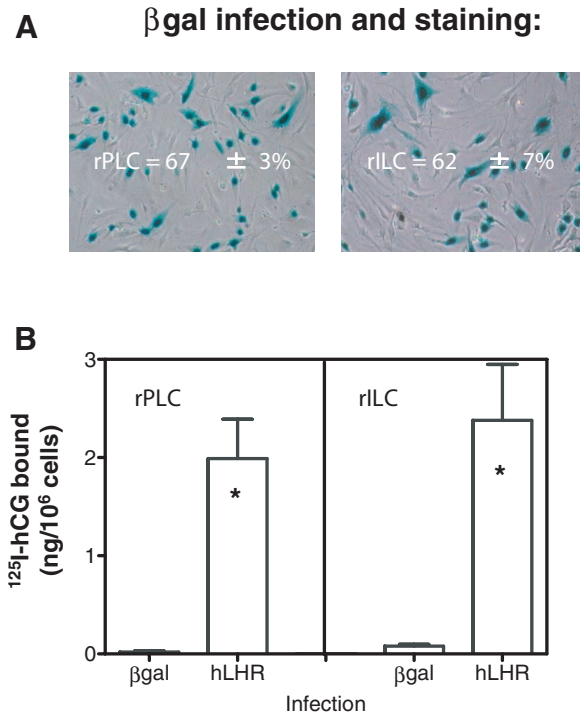

FIG. 2. Recombinant proteins can be readily expressed in primary cultures of rat Leydig cells by infection with adenoviral vectors. A, Two days after infection with Ad-βgal, the cells were stained for βgal activity and examined by phase contrast microscopy as shown. The percentage of stained cells was scored as described in *Materials and Methods*. The micrographs show the results of one representative experiment, and the numbers inside them show the percentage of infected cells as judged by βgal staining (mean ± SEM of three independent infections). B, ^{125}I-hCG binding was measured 2 d after infection with Ad-βgal or Ad-hLHR during a 1-h incubation at room temperature with 100 ng/ml ^{125}I-hCG as described in *Materials and Methods*. Each *bar* shows the mean ± SEM of three independent infections. *, $P < 0.05$, statistical significance when compared with same cell stage infected with Ad-βgal (two-tailed *t* test).

70% and the binding of ^{125}I-hCG to approximately 6 ng/10^6 cells (data not shown). All experiments shown herein were done with cells infected with Ad-hLHR at an MOI of 200.

Although the hLHR construct subcloned into the adenoviral vector is tagged with the myc epitope (44), we cannot detect its expression by fluorescent microscopy using anti-myc antibodies[1]. This is likely attributable to the relatively low level of expression of the receptor. In our experience, this method allows for detection of receptor expression in transiently transfected cells only at levels of ^{125}I-hCG binding that are three to five times higher than that shown in Fig. 2B (42). Thus, we do not really know what percentage of the cells infected with the Ad-hLHR express the recombinant hLHR. We can only approximate this number to 65% based on the data obtained with the Ad-βgal-infected cells (Fig. 2A).

In agreement with the lack of ^{125}I-hCG binding in the cells that were not infected with the Ad-hLHR, classical hCG responses such as cAMP synthesis, inositol phosphate accumulation, and steroid synthesis were either undetectable or barely detectable in the cells infected with Ad-βgal (Fig. 3).

[1] Commercially available antibodies to the LHR are not better for these assays than antibodies to the myc epitope.

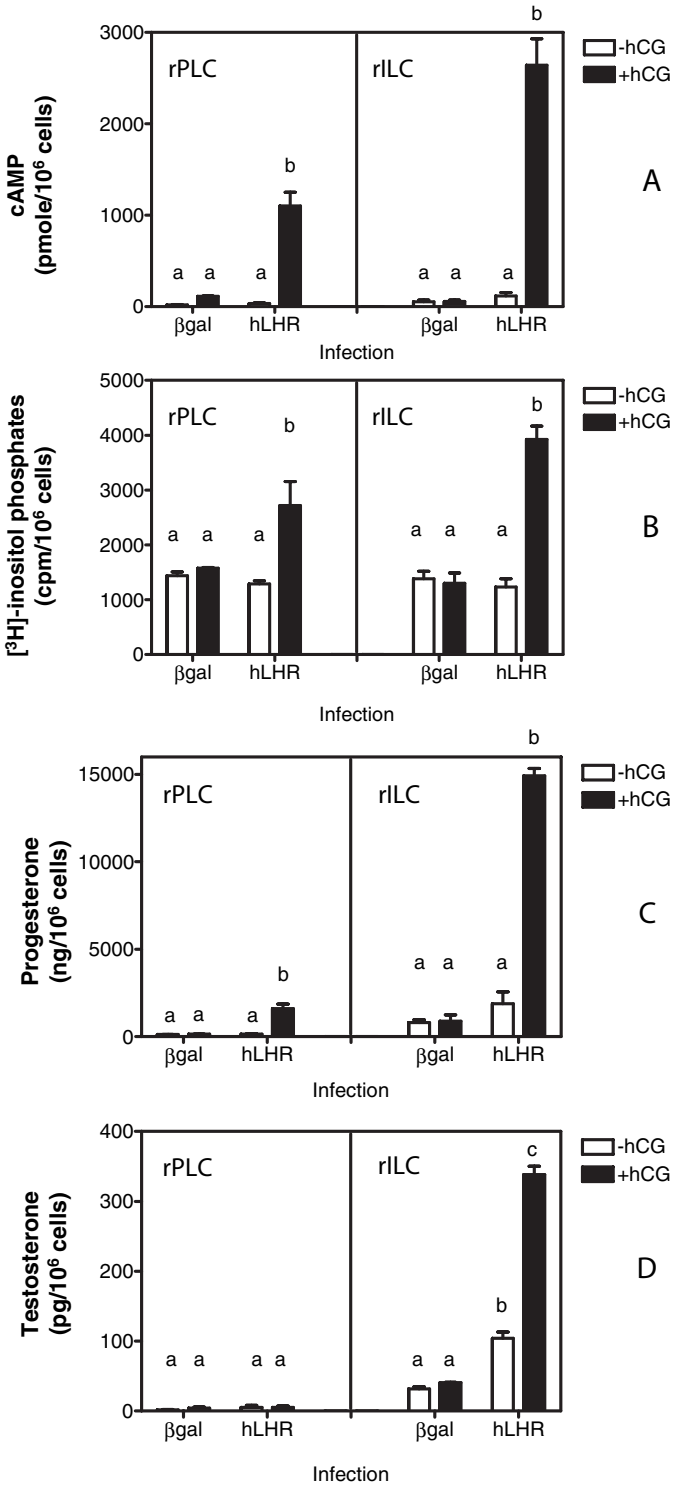

FIG. 3. Expression of the hLHR allows hCG to stimulate cAMP, inositol phosphate, and steroid accumulation in primary cultures of rat Leydig cells. The cells were infected on d 2 with Ad-βgal or Ad-hLHR (200 MOI) as indicated. All assays were performed on d 4 in cells incubated without or with 500 ng/ml hCG for 1 h (inositol phosphates; B) or with 100 ng/ml hCG for 4 h (cAMP and steroid assays; A, C, and D) as described in *Materials and Methods*. Each *bar* is the mean ± SEM of three to five independent experiments. Means within a panel with *different letters* (a, b, c) are significantly different from each other ($P < 0.05$, ANOVA with Bonferroni's *post hoc* test).

In contrast, addition of hCG to Ad-hLHR-infected cells robustly stimulated cAMP (Fig. 3A), inositol phosphates (Fig. 3B), and steroid accumulation (Fig. 3, C and D). The steroid responses shown in Fig. 3, C and D, are interesting because they show that rPLCs infected with the Ad-hLHR respond to hCG with an increase in progesterone synthesis, whereas rILCs respond with an increase in both progesterone and testosterone synthesis. The levels of progesterone detected in the immature cells are much higher than the levels of testosterone, however (Fig. 3, compare the scales in C and D). We did not examine the reasons for the lack of a testosterone response in the rPLCs, but others have shown that, at 20–40 d postpartum, the maturing Leydig cells produce mostly 5α-reduced androgens rather than testosterone (for review, see Ref. 46). We also note that the increased levels of testosterone in the immature cells are in agreement with the higher expression of 3β-HSD in this cell stage (Fig. 1B).

Lastly, although not always obvious from the data presented in Fig. 3, the basal levels of cAMP, progesterone, and testosterone tended to be higher in Ad-hLHR-infected than in Ad-βgal-infected cells. This trend attained statistical significance only for testosterone synthesis in the immature cells (Fig. 3D).

Activation of the LHR stimulates ERK1/2 phosphorylation in primary cultures of rPLCs and rILCs

Because we wanted to determine whether the ERK1/2 cascade is involved in the proliferative response of Leydig cells, we also examined the ability of hCG to increase the phosphorylation of ERK1/2 in these primary cultures. Figure 4 shows that Ad-βgal-infected rPLCs or rILCs do not respond to hCG with a significant increase in ERK1/2 phosphorylation. Infection with Ad-hLHR did not affect basal phosphorylation of ERK1/2, but it conferred robust hCG responsiveness to both cell stages. Figure 4 also shows that the ERK1/2 pathway of both cell stages is sensitive to epidermal growth factor (EGF) stimulation regardless of the expression of the hLHR. EGF was used as a positive control in this and subsequent experiments because activation of the EGF receptor (EGFR) stimulates the ERK1/2 cascade in MA-10 cells (26, 27) and DNA synthesis in primary cultures of rat Leydig cells (38, 40, 41). Moreover, EGF is a potent mitogen for many cell types (48).

When consider together, the data summarized in Figs. 3 and 4 show that 4-d-old primary cultures of Leydig cells do not retain robust responsiveness to hCG unless they are infected with Ad-hLHR. We note, however, that there may be some residual hCG-induced responses in progenitor and immature cells that were not infected with Ad-hLHR. hCG-induced cAMP responses in rPLC infected with Ad-βgal (Fig. 3A, *left*) and the hCG-induced ERK1/2 responses of rPLC and rILC infected with Ad-βgal (Fig. 4, *left column*) appeared higher than those of cells incubated without hCG but did not attain statistical significance. The reason why only some responses are measurable is not known, but it could simply be attributable to the presence of a small amount of endogenous receptors and the sensitivity of the different assays used. Moreover, even when these responses were present, they were clearly enhanced by infection with Ad-hLHR (Figs. 3 and 4).

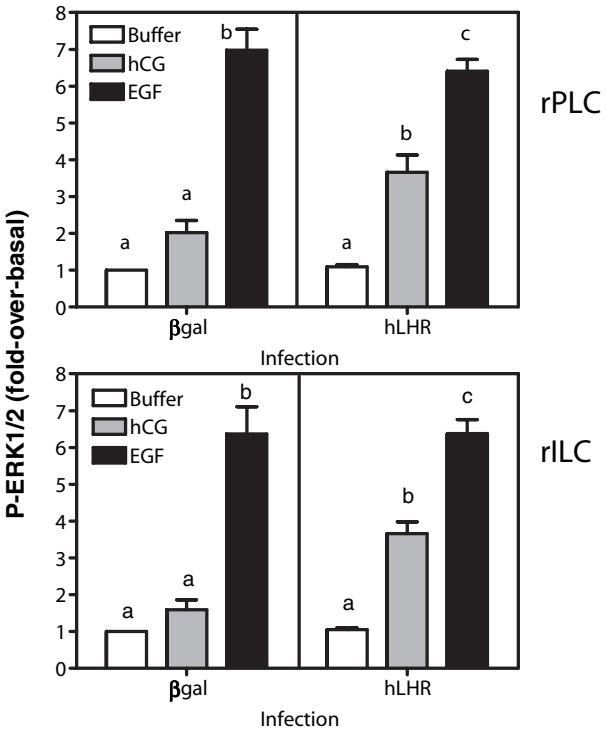

FIG. 4. Expression of the hLHR allows hCG to stimulate ERK1/2 phosphorylation in primary cultures of rat Leydig cells. The cells were infected on d 2 with Ad-βgal or Ad-hLHR (200 MOI) as indicated. The phosphorylation of ERK1/2 was measured on d 4 in cells incubated with buffer only, 100 ng/ml hCG (for 15 min), or 100 ng/ml EGF (for 5 min) as indicated and as described in *Materials and Methods*. The results are presented as fold over basal, and each *bar* is the mean ± SEM of four to seven independent experiments. Means within a panel with *different letters* (a, b, c) are significantly different from each other ($P < 0.05$, ANOVA with Bonferroni's *post hoc* test).

The ability of hCG to enhance ERK1/2 phosphorylation in Leydig cells has been studied previously using freshly isolated rILCs or MA-10 cells (26–28). This pathway appears to require the participation of protein kinase A (PKA), Fyn, the EGFR, and Ras. Some of these conclusions were confirmed by the experiments presented in Figs. 5 and 6[2]. Figure 5 shows that ERK1/2 phosphorylation in primary cultures of rPLCs or rILCs can be stimulated with 8-CPT-cAMP, a cAMP analog that does not discriminate between PKA and the Epacs (exchange protein directly activated by cAMP) or with N^6-Benzoyl-cAMP, which is selective for PKA, but it cannot be stimulated with 8-CPT-2Me cAMP, which is Epac selective (49–51). In agreement with the data obtained in MA-10 cells (27), the results presented in Fig. 6 (*top row*) show that the hCG-induced phosphorylation of ERK1/2 in primary cultures of rPLCs or rILCs infected with the Ad-hLHR can be partially blocked with an inhibitor of the Src family of kinases (PP2) or an inhibitor of the EGFR kinase (AG1478). The

[2] The concentrations of cAMP analogs and inhibitors shown in Figs. 5 and 6 are the lowest concentrations that elicit maximal activation (cAMP analogs) or the lowest concentrations that elicit a maximal inhibitory effect (chemical inhibitors). These concentrations were chosen empirically (data not shown).

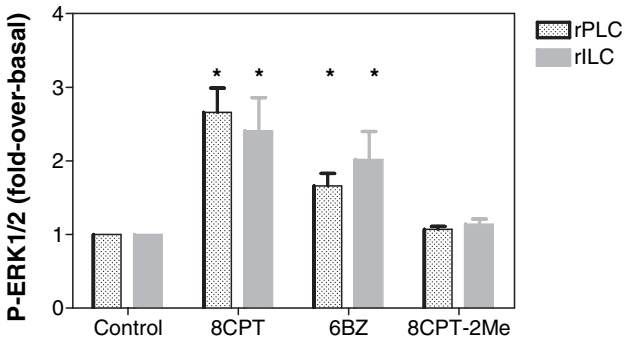

FIG. 5. A PKA-selective cAMP analog stimulates ERK1/2 phosphorylation in primary cultures of rat Leydig cells. The cells were infected on d 2 with 200 MOI Ad-hLHR. The phosphorylation of ERK1/2 was measured on d 4 in cells incubated with buffer only, 0.5 mM 8-CPT-cAMP (8CPT), 6-Benzoyl-cAMP (6BZ), or 8-CPT-2Me cAMP (8CPT-2Me) for 15 min as indicated and as described in *Materials and Methods*. The results are presented as fold over basal, and each *bar* is the mean ± SEM of three independent experiments. *, $P < 0.05$, significant differences when compared with same stage cells incubated with buffer only (control) (ANOVA with Dunnett's *post hoc* test).

inactive analogs of these compounds (PP3 and AG43, respectively) have no effect. These data also show that U0126 and PD98059, two inhibitors of the MAPK kinase MEK (the kinase that phosphorylates ERK1/2), completely block the ability of hCG to stimulate ERK1/2 phosphorylation. Lastly, GM6001, a broad metalloprotease inhibitor that blocks the proteolysis of some of the EGF-like growth factor precursors (52) has no effect on hCG-induced ERK1/2 phosphorylation. The same inhibitors were tested on the EGF-stimulated ERK1/2 phosphorylation, and the results (Fig. 6, *bottom row*) are in complete agreement with those reported previously using MA-10 cells as well (27). The EGF-provoked ERK1/2 response is inhibited only by the inhibitor of the EGFR kinase (AG1478) and by the two MEK inhibitors (U0126 and PD98059).

hCG and EGF are mitogens for primary cultures of rPLCs and rILCs

To determine whether activation of the LHR promotes the proliferation of Leydig cells, we first attempted to examine this issue by measuring [^{3}H]thymidine incorporation in freshly isolated rPLC or rILC as described by others (36, 38–41).

In agreement with previous results (39), we found that freshly isolated rPLCs have a higher basal level of DNA synthesis than freshly isolated rILCs (Fig. 7A) and that there is a spontaneous decrease in basal levels of DNA synthesis (36) as the cells are maintained in culture for 2 d (Fig. 7, compare the *open columns* for each cell type in A and B). Others have previously examined the effects of hCG or growth factors such as EGF or IGF-I on [^{3}H]thymidine incorporation in freshly isolated rPLCs or rILCs (that have been allowed to become quiescent by serum deprivation for 24 h) with mixed success. The growth factors always significantly stimulate thymidine incorporation in these cultures, but the effects of hCG are not always significant (38–41).

Using uninfected rPLCs or rILCs, we found that hCG did not stimulate [^{3}H]thymidine incorporation (Fig. 7B). Under the same conditions, EGF had a small stimulatory effect, however (Fig. 7B). It should also be noted that the effect of EGF, although statistically significant, was of small magnitude. Lastly, neither hCG nor EGF increased the number of cells attached to the wells. These findings agree with the lack of many other LHR-mediated responses in primary cultures of noninfected rPLCs or rILCs (Figs. 3 and 4).

Because the culture conditions used above cannot be used to study the possible effects of hCG on Leydig cell proliferation, we next tried Ad-hLHR-infected cells. Subconfluent cultures of Ad-hLHR-infected rPLCs or rILCs were incubated with buffer only, hCG, or EGF. The number of attached cells were measured after 24 or 48 h (Fig. 8, *top row*), and the rates of DNA synthesis were measured using a [^{3}H]thymidine pulse given during the last 4 h of the initial 24-h incubation (Fig. 8, *bottom row*). When incubated in the absence of hormones, subconfluent cultures of Ad-hLHR-infected cells maintained a constant cell density for 2 d. A 2-d incubation with hCG or EGF increased the density of rPLCs 1.9- and 2.1-fold, respectively. hCG or EGF also increased the density of rILCs 1.4- and 1.6-fold, respectively (Fig. 8, *top row*). Most or all of the increase in cell density provoked by hCG or EGF occurred during the first day of addition of the hormones. This is likely attributable to the inability of the primary cultures to continue to proliferate rather than to a limitation imposed by contact inhibition. Cultures containing half as many cells as those used for the experiment shown in Fig. 8 also failed to increase in density when the incubation with hCG and EGF was continued beyond 2 d.

To ensure that hCG and EGF were stimulating the proliferation of Leydig cells rather than a contaminating cell population, we stained the primary cultures for 3β-HSD before and after a 2-d incubation with hCG or EGF. The results of these experiments showed that, at the end of the 2-d incubation with hCG or EGF, the percentage of cells staining positively for 3β-HSD [*i.e.* >95% (Fig 1A)] was similar to that detected on d 4 before the hormones were added (data not shown).

Addition of hCG or EGF significantly increased [^{3}H]thymidine incorporation in both cell types (Fig. 7, *bottom row*), but the magnitude of these effects was somewhat higher in rPLCs (3.4- and 10.9-fold for hCG and EGF, respectively) than in rILCs (2.8- and 7.7-fold for hCG and EGF, respectively).

Whereas the stimulatory effects of hCG and EGF on cell density were not statistically different (Fig. 9, *top row*), EGF was more effective than hCG in stimulating thymidine incorporation (Fig. 8, *bottom row*). This could be attributable to differences in the sensitivity of the assays or to the stimulation of opposing pathways. For example, hCG could be stimulating only DNA synthesis, whereas EGF could be stimulating DNA synthesis and apoptosis. Thus, in the case of EGF, a higher rate of cell multiplication could be balanced by a higher rate of cell death. Although the reasons for these differences remain unresolved, both assays show that hCG and EGF are mitogenic stimuli for Leydig cells of these two stages of differentiation.

Lastly, it should be noted that, although basal levels of DNA synthesis decline as the cells are maintained in culture, the basal level of DNA synthesis is always higher in rPLCs

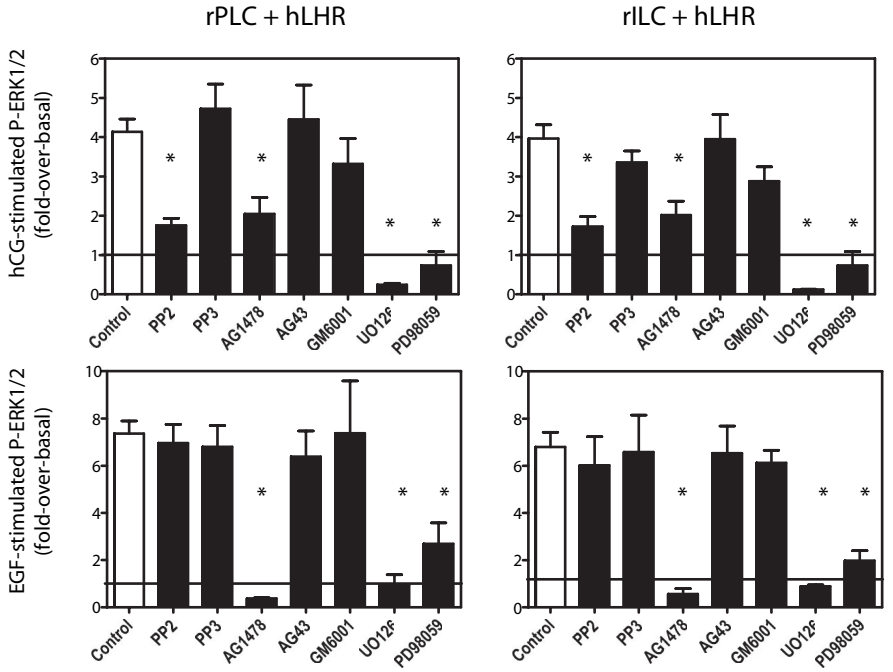

FIG. 6. Effects of several inhibitors on the hCG- or EGF-induced ERK1/2 phosphorylation in primary cultures of rat Leydig cells. The cells were infected on d 2 with 200 MOI Ad-hLHR. On d 4, the cells were preincubated for 30 min with PP2 or PP3 (10 μM), AG1478 or AG43 (1 μM), GM6001 (2 μM), U0126 (10 μM), or PD98059 (100 μM) as indicated. The phosphorylation of ERK1/2 was then measured at the end of a 15-min incubation with 100 ng/ml hCG (*top row*) or a 5-min incubation with 100 ng/ml EGF (*bottom row*). The results are presented as fold over basal, and each *bar* is the mean ± SEM of three independent experiments. *, $P < 0.05$, significant differences when compared with same stage cells incubated with buffer only (control) (ANOVA with Dunnett's *post hoc* test). The horizontal line is drawn at a value of 1, which is the basal level of ERK1/2 phosphorylation.

than in rILCs. [^{3}H]Thymidine incorporation levels in freshly isolated, 2-d-old uninfected, and 5-d-old Ad-hLHR-infected rPLCs were 2016 ± 141, 681 ± 56, and 430 ± 59 cpm/10^5 cells, respectively. The corresponding values for rILCs were 784 ± 45, 263 ± 35, and 212 ± 16.

The ERK1/2 cascade is involved in the LHR-promoted proliferation of rPLCs and rILCs

To determine whether the ERK1/2 cascade is a mediator of the effects of hCG on Leydig cell proliferation, we decided to test the effects of two MEK inhibitors (U0126 and PD98059) and one inhibitor of the Src family of kinases (PP2) on the ability of hCG to stimulate the multiplication of rPLCs or rILCs. These inhibitors were chosen based on the data presented in Fig. 6 showing that U0126 and PD98059 inhibit the hCG- or EGF-stimulated ERK1/2 phosphorylation, whereas PP2 inhibits only the hCG-mediated response (Fig. 6).

Because the ERK1/2 phosphorylation assays are done with a time frame of several minutes and cell proliferation assays are done over a period of 24–48 h (Figs. 7 and 8), we first incubated the primary cultures or rPLCs or rILCs with each of these compounds [at the concentrations known to inhibit ERK1/2 phosphorylation (Fig. 6)] for 2 d and examined them by microscopy to determine whether there were overt signs of toxicity. After a 2-d incubation with U0126, rILCs had a normal morphology, whereas those incubated with PD98059 or PP2 showed distinct signs of toxicity such as cell rounding, detachment, and other overt changes in cell morphology (data not shown).

Based on these observations, we decided to use only U0126 to determine whether the ERK1/2 cascade is involved in the proliferative response of Leydig cells to EGF or hCG. We incubated the cells with or without U0126 and stimulated them with hCG or EGF. The number of cells was determined after a 48-h incubation, and DNA replication was measured during the last 4 h of a 24-h incubation. The results of these experiments (Fig. 9) clearly show that U0126 is an effective inhibitor of the hCG- or EGF-induced proliferation of Leydig cells.

Two additional indexes of cell multiplication, increased expression of cyclin D3 and quantitation of viable cells by measuring mitochondrial function, also revealed that EGF and hCG stimulate cell multiplication through an ERK1/2-dependent pathway (data not shown).

Discussion

The studies summarized here were designed to establish a Leydig cell culture model that could be used to study the effects of LH/CG on the proliferation of Leydig cells and the signaling pathways that participate in this process. Because rPLCs and rILCs found in the postnatal testis have the capacity to proliferate (46, 47), we focused our attention on these two cell populations. In agreement with the results of others (28, 31–33), we were able to prepare primary cultures of homogenous populations or rat Leydig cells of these two different stages of differentiation. After 4 d in culture, however, these cells do not bind ^{125}I-hCG or respond robustly to hCG with any of the classical hCG-induced responses (such as cAMP, inositol phosphate, and steroid synthesis) unless they are first infected with an Ad coding for the hLHR. After 1 d in culture, uninfected cells also failed to respond to hCG with an increase in DNA synthesis. At first glance, these findings seem different than those of others who have been able to maintain steroidogenic responses in primary cultures of rPLCs or adult rat Leydig cells attached to Cytodex beads for 3 d (32, 34, 35). This has been accomplished, however, only under chronic hormonal stimulation because these cultures were maintained in medium supplemented with low levels of partially purified LH either alone or together with DHT from the beginning of the culture period (32, 34, 35). The

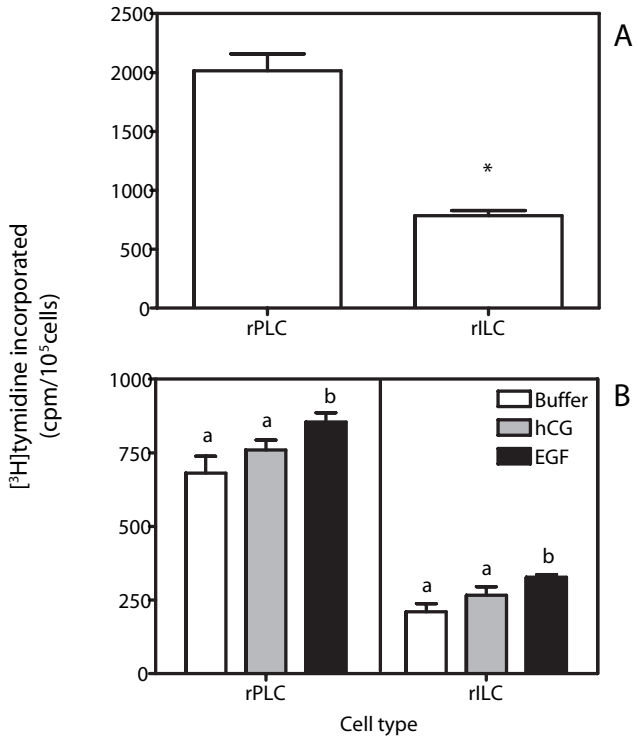

FIG. 7. hCG does not stimulate DNA replication in primary cultures of rat Leydig cells that are not infected with Ad-hLHR. A, One milliliter of freshly isolated cells containing 1–2 $\times$ 10^5 progenitor or immature cells (kept in suspension) were incubated for 4 h with [^{3}H]thymidine. Incorporated thymidine was then measured as described in *Materials and Methods*. Each *bar* represents the mean $\pm$ SEM of four independent experiments. *, $P < 0.05$, statistical significance between the two cell stages (paired *t* test). B, Freshly isolated rPLCs or rILCs (1–2 $\times$ 10^5) were plated on a 12-well plate as described in *Materials and Methods*. Twenty-four hours after plating, the medium was changed and the cells were incubated in 1 ml of serum-free medium containing buffer only, hCG, or EGF (each at 100 ng/ml) as indicated. Twenty hours later, [^{3}H]thymidine was added and the incubation was continued for 4 h. Incorporated thymidine was then measured as described in *Materials and Methods*. Parallel cultures were used to determine cell density at the beginning and at the end of the 24-h incubation with buffer or hormones. The number of attached cells varied from 0.7 to 1.8 $\times$ 10^5 per well, but it did not change from the beginning to the end of the 24-h incubation. The number of cells was also the same in the cultures incubated with buffer only, hCG, or EGF. Each *bar* is the mean $\pm$ SEM of four independent experiments. Within a panel, the *bars* with *different letters* (a, b) are significantly different from each other ($P < 0.05$, ANOVA with Bonferroni's *post hoc* test).

conditions used here are different in that our primary cultures of Leydig cells are maintained attached to gelatin-covered cell culture plasticware and in the absence of LH or DHT. We do not consider this lack of responsiveness a hindrance because Leydig cells can be readily infected using adenoviral vectors, and, thus, responsiveness to hCG can be restored by infection with an Ad coding for the hLHR. Under the conditions chosen here, about 65% of the cells can be infected and the level of ^{125}I-hCG binding attained corresponds to approximately 25,000 LHR per cell (assuming that all cells express the hLHR) or approximately 38,000 LHR per cell (assuming that only 65% of the cells express the hLHR). This LHR density is similar to that of the endogenous LHR expressed in freshly isolated rILCs or adult rat Leydig cells that have been reported to express approximately 9000 and 20,000–40,000 receptors per cell (32, 34). The LHR density in freshly isolated rPLCs is only approximately 2500 receptors per cell, however (34).

As already mentioned (see *Results*), we can, to some extent, modulate the density of hLHR expressed in these cultures by changing the amount of virus used for infections. Here we chose to use a single receptor density because we already know from doing these types of experiments with MA-10 cells that increasing the density of the hLHR expressed increases the magnitude of the hCG-induced responses and their sensitivity to hCG (42). Increasing the density of the hLHR expressed in MA-10 cells also increases the basal levels of second messengers and steroid synthesis. This is attributable to the presence of an intrinsic (agonist-independent) activity of G protein-coupled receptors in general (53) and the hLHR in particular (6, 42). A similar trend was apparent in the primary cultures, but this trend attained statistical significance only on basal levels of testosterone in the immature cells. This difference is likely attributable to the lower density of recombinant hLHR expressed in the primary cultures (approximately 25,000 per cell; see above) than in MA-10 cells (approximately 100,000 per cell) (42).

We choose to express the hLHR instead of the rLHR because the expression of the recombinant hLHR is better than that of the recombinant rLHR (6). In fact, although expression of the recombinant rodent LHR can be readily detected in heterologous cell types that robustly express recombinant proteins [such as HEK293 or COS-7 cells (see Ref. 6)], we have not been able to detect expression of the rodent LHR by transfection of target cells such as MA-10 cells. In addition, a large number of studies conducted by many different investigators have shown that, when expressed in heterologous cell types, the recombinant rodent and hLHR activate the same second-messenger pathways such as cAMP and inositol phosphate accumulation (for review, see Ref. 6), and a detailed study from our laboratory shows that expression of increasing amounts of the hLHR in MA-10 cells (a mouse Leydig cell line that expresses a low level of endogenous LHR) simply enhances the magnitude and increases the sensitivity of these cells to hCG-induced responses (such as cAMP accumulation, steroidogenesis, and ERK1/2 activation) that are also provoked by the endogenous LHR (42). Lastly, there are a large number of activating and inactivating mutants of the hLHR associated with Leydig cell hyperplasia, Leydig cell adenomas, or Leydig cell hypoplasia (for review, see Refs. 6 and 7) that could be used in future experiments designed to determine their effects on these cultures.

Because of our interest in Leydig cell proliferation, we explored the hCG-induced ERK1/2 response in some detail. We have shown previously that hCG induces ERK1/2 phosphorylation in MA-10 cells by a pathway that requires PKA, Fyn, EGFR, and Ras activation (26, 27), but, because they are transformed, MA-10 cells are not a good model to study the proliferative effects of hCG and the pathways that mediate these effects. Others have also documented an hCG-induced and PKA-dependent ERK1/2 response in freshly isolated rILCs, but the potential involvement of this pathway on

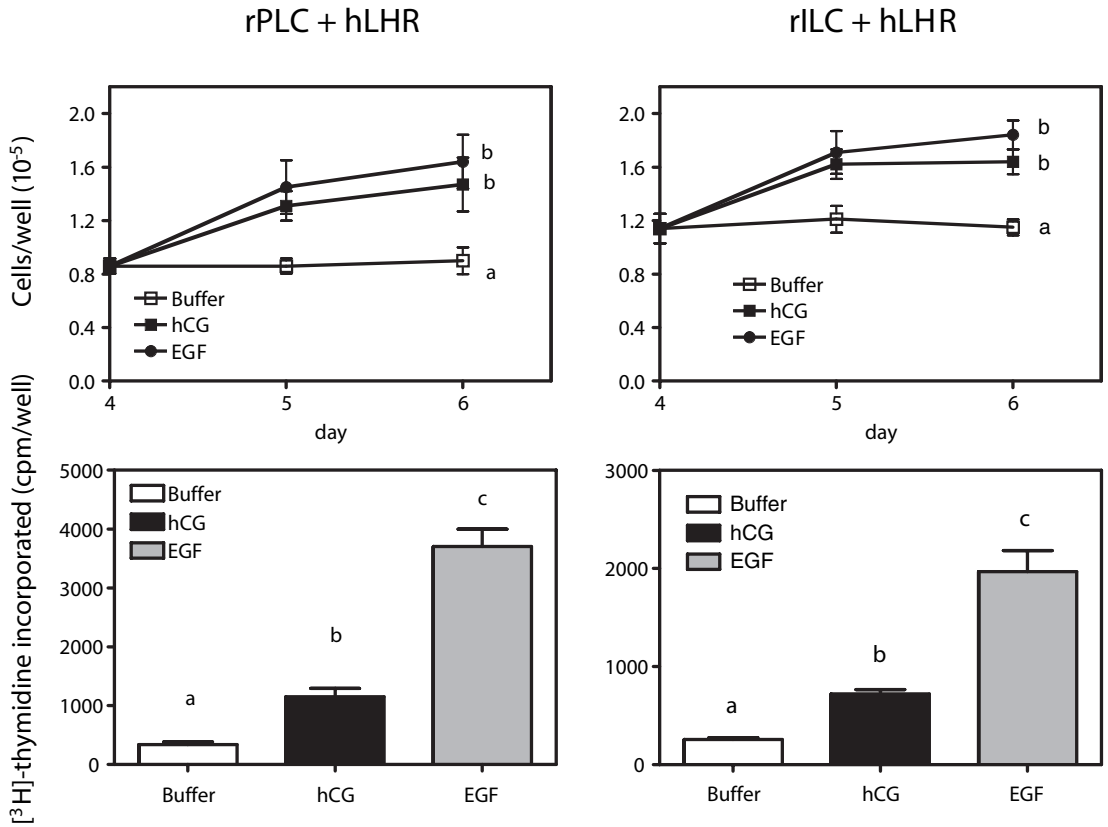

FIG. 8. hCG and EGF stimulate the proliferation of primary cultures of rat Leydig cells. The cells were infected on d 2 with 200 MOI Ad-hLHR. *Top row*, On d 4, subconfluent wells were cultured in serum-free medium containing buffer only, hCG (100 ng/ml), or EGF (100 ng/ml) as indicated. The medium and hormones were replaced on d 5. The plates were trypsinized and the number of cells was determined on d 4, 5, and 6 as described in *Materials and Methods*. Each *point* is the mean ± SEM of six independent experiments. Statistical analysis was performed only for the data obtained on d 6. Within a panel, the d 6 points with *different letters* (a, b) are significantly different from each other ($P < 0.05$, ANOVA with Bonferroni's *post hoc* test). *Bottom row*, On d 4, subconfluent wells were cultured in serum-free medium containing buffer only, hCG (100 ng/ml), or EGF (100 ng/ml) as indicated. The cells were labeled with [^{3}H]thymidine during the last 4 h of a 24-h incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each *bar* is the mean ± SEM of six independent experiments. Within a panel, the values of the *bars* marked by the *different letters* (a, b, c) are significantly different from each other ($P < 0.05$, ANOVA with Bonferroni's *post hoc* test). Note the different vertical scales in the *left* and *right panels*.

Leydig cell proliferation was not investigated (28). In agreement with these previously published data, the results presented here show that hCG and a PKA-selective cAMP analog provoke the phosphorylation of ERK1/2 in rPLCs and rILCs and that the effects of hCG are sensitive to inhibitors of MEK, the Src family of kinases, or the EGFR kinase. Likewise, in agreement with the data obtained with MA-10 cells (27), we show that the effect of hCG on ERK1/2 phosphorylation in the rILCs and rPLCs is not sensitive to a metalloprotease inhibitor.

In our view, the most interesting findings reported here are 1) that primary cultures of rPLCs or rILCs expressing the hLHR proliferate in response to hCG stimulation; and 2) that this response is sensitive to an inhibitor of ERK1/2 phosphorylation. The proliferative response to hCG is rather robust. Counting cells and staining the cultures for 3β-HSD (an index of the homogeneity of the cell population) show that the number of rPLCs or rILCs almost doubles during a 24- to 48-h incubation period in response to hCG. Remarkably, this hCG-provoked increase in cell density is similar to the increase in cell density elicited by EGF, a potent and well-characterized mitogen for many cell types. We do not yet know whether hCG is a direct mitogen for Leydig cells or whether it induces their proliferation by activating an autocrine/paracrine loop involving stimulation of the synthesis and/or release of growth factors such as IGF-I (36, 54) or members of the EGF family (55, 56).

In agreement with the results of others (39), we find that DNA synthesis is higher in freshly isolated rPLCs than freshly isolated rILCs and that the basal level of DNA synthesis declines as cells are placed in culture (36). Interestingly, however, even after 5 d in culture, the relative rate of DNA synthesis under basal conditions is higher in the progenitor cells. This is an important finding that mimics the proliferative properties of these two cell stages in the whole animal (46, 47). After 4 d in culture, hCG stimulated DNA synthesis 3- to 4-fold, whereas EGF stimulated DNA synthesis 8- to 10-fold in the Ad-hLHR-infected rPLCs or rILCs. The finding that the proliferative effects of EGF and hCG, measured by cell counting or by [^{3}H]thymidine incorporation, are sensitive to an MEK inhibitor show that the ERK1/2 cascade is a mediator of the proliferative response of Leydig cells. The ERK1/2 cascade is, of course, a prominent mitogenic cascade (57, 58) that is known to be sensitive to LHR

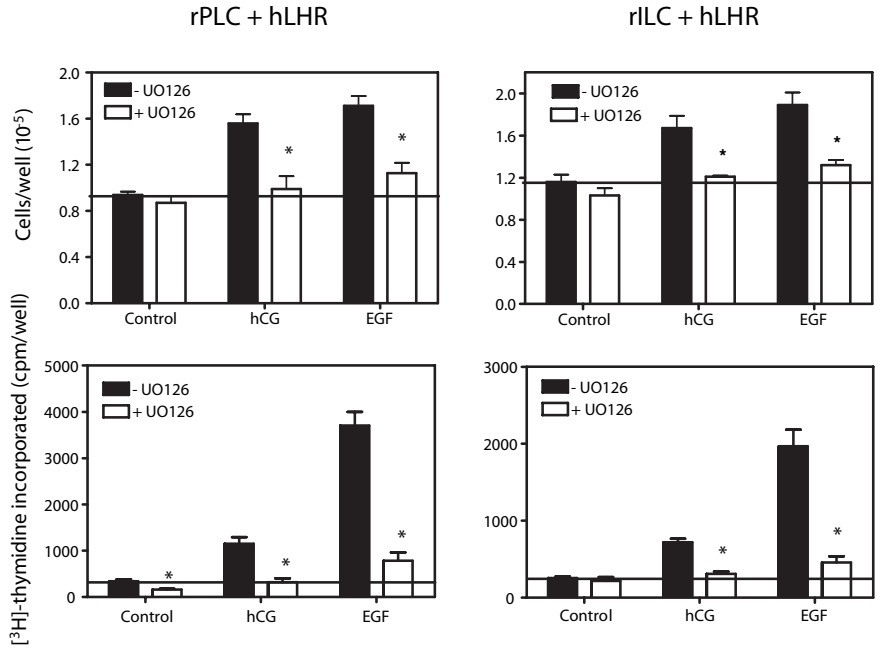

FIG. 9. U0126 inhibits the proliferative effects of hCG and EGF on primary cultures of rat Leydig cells. The cells were infected on d 2 with 200 MOI Ad-hLHR. *Top row*, On d 4, subconfluent wells were cultured in serum-free medium without or with 10 μM U0126 containing buffer only, hCG (100 ng/ml), or EGF (100 ng/ml) as indicated. The medium, inhibitor, and hormones were replaced on d 5. The plates were trypsinized and the number of cells was determined on d 6 as described in *Materials and Methods*. Each *bar* is the mean ± SEM of six independent experiments. For a given bar, the *asterisks* denote significant differences when compared with cells incubated without U0126 for a given pair ($P < 0.05$, two-tailed *t* test). The *horizontal line* is drawn at a value of 1. This value marks the number of cells present at the beginning of the experiment (d 4). *Bottom row*, On d 4, subconfluent wells were cultured in serum-free medium without or with 10 μM U0126 containing buffer only, hCG (100 ng/ml), or EGF (100 ng/ml) as indicated. The cells were labeled with [^{3}H]thymidine during the last 4 h of a 24-h incubation, and the radioactivity incorporated into DNA was measured as described in *Materials and Methods*. Each *bar* is the mean ± SEM of six independent experiments. For a given pair, the *asterisks* denote significant differences ($P < 0.05$, two-tailed *t* test) when compared with cells incubated without U0126. The *horizontal line* is drawn at the level of [^{3}H]thymidine incorporated into control cells incubated without U0126. Note the different vertical scales in the *left* and *right panels*.

activation in Leydig cells (26–28). Up until now, however, the ERK1/2 pathway has been implicated only in the regulation of steroidogenesis in Leydig cells (28, 59). Its potential involvement in Leydig cell multiplication has not been examined.

Although there is abundant evidence for a role of LH/CG and the LHR in the proliferation and differentiation of Leydig cells (see introductory section), the molecular basis of the proliferative effects of LH/CG on Leydig cells are not understood. A common whole-animal model used to study Leydig cell proliferation is the regeneration of rat Leydig cells that occurs after their selective destruction with ethane dimethane sulfonate (60). Studies using this model have indeed shown that, after ethane dimethane sulfonate ablation, the process of Leydig cell regeneration is regulated by LH (61, 62), but the mechanisms by which LH promotes Leydig cell regeneration have not been studied. Although we are not aware of any reports in which the effects of LH/CG on the proliferation of primary cultures of Leydig cells have been examined, there are a number of reports on the effects of LH/CG on DNA replication in these cultures. The effects of LH/CG on [^{3}H]thymidine incorporation in cultures of rILCs or rPLCs maintained for 24 h in the absence of LH are weak and not always significant (38–41). Moreover, maintaining the primary cultures of rPLCs or rILCs with a low level of partially purified LH for 48 h [conditions that are known to maintain steroidogenesis (see Refs. 32, 34, and 35)] was shown to induce a small increase in [^{3}H]thymidine incorporation but had no effect on the subsequent response to a higher concentration of LH/CG (38, 41).

The studies presented here with primary cultures expressing the recombinant hLHR serve to establish a novel experimental paradigm in which the effects of LHR activation on Leydig cell proliferation and DNA replication can be readily studied in cell culture. We suggest that the rILCs (instead of the rPLCs) are the best model to study the proliferative response of Leydig cells to LH/CG for four reasons: 1) the Leydig cell lineage of this cell stage can be more clearly discerned as indicated by their testosterone response to hCG stimulation; 2) the density of recombinant hLHR expressed in these cells is similar to that measured in freshly isolated cells; 3) their proliferative response to hCG is only slightly lower than that of rPLCs; and 4) they are isolated from the postnatal period that corresponds to development of the adult generation of Leydig cells when the levels of LH and testosterone are both rising (63).

Lastly, although our studies were restricted to expression of the recombinant wild-type hLHR, our data show that adenoviral vectors can be readily used to express recombinant proteins in primary cultures of rat Leydig cells. This method should thus be useful in the study of the actions of naturally occurring mutants of the hLHR or any other aspect

of the biology of Leydig cells that is facilitated by the expression of recombinant proteins.

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Address all correspondence and requests for reprints to: Mario Ascoli, Ph.D., Department of Pharmacology, 2-319B BSB, 51 Newton Road, The University of Iowa, Iowa City, Iowa 52242. E-mail: mario-ascoli@uiowa.edu.

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