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Progesterone Action in a Murine Leydig Tumor Cell Line (mLTC-1), Possibly through a Nonclassical Receptor Type*

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ABSTRACT

In a recent report we demonstrated that a high (micromolar) concentration of progesterone (P) specifically down-regulates LH receptor (R) expression and function in murine Leydig tumor cells. The aim of the present study was to characterize further the putative novel R, mediating these P effects in the murine Leydig tumor cell line, mLTC-1. The binding of [^{3}H]P to these cells revealed a high (K_d, ~9.3 nmol/liter) and a low affinity (K_d, ~284 nmol/liter) component, and the binding displayed with specificity (P > dehydroepiandrosterone > 17-OHP). The binding was apparently different from that of the classical nuclear PR in the following ways. 1) The P/glucocorticoid antagonist RU 486 did not compete with [^{3}H]P binding to the mLTC-1 cells. 2) No expression of the classical PR messenger RNA was detected, despite clear P binding to these cells, by Northern hybridization or RT-PCR. 3) An antibody against the C-terminal end of the classical PR (αc-262) revealed in mLTC-1 cells several molecular size protein bands between 45–57 kDa on Western hybridization, whereas these immunoreactive proteins were faintly recognized by another antibody (α-PR) directed toward the NH_2-terminal region of the classical PR. The sizes of the immunoreactive molecules were relatively similar to those detected using the same antibodies in human sperm lysates, but were at variance with the classical PR (120, 94, and 60 kDa), detected with these antibodies in human uterus. The immunoreactive proteins bound peroxidase-labeled-P, which could be displaced in the presence of a 10-fold excess of free P. 4) An immediate increase in the intracellular free calcium level was observed after P treatment in cultured mLTC-1 cells, whereas it also increased the $^{45}Ca^{2+}$ entry within 15 min in these cells. 5) Increasing doses of P (0.1–10 μmol/liter) demonstrated significant inhibition of LH receptor messenger RNA levels in a dose-dependent manner in mLTC-1 cells. In conclusion, a nonclassical PR is expressed and functional in these cells, and it is clearly distinct from the classical nuclear PR. It is apparent that recently reported inhibitory effects of P on LH receptor gene expression and function are mediated through this novel type PR in mouse Leydig cells. (*Endocrinology* **141:** 247–255, 2000)

THE CLASSICAL PATHWAY of progesterone (P) actions involves binding of the hormone in target cells to a specific nuclear receptor (R). The ligand-activated PR, thereafter, acting as a transcription factor, binds to specific regulatory sequences of responsive target genes, resulting in their transcriptional regulation (1). Previous studies demonstrate the existence of alternative forms of PR that differ in structure and mechanisms of action from the classical nuclear receptor (2–4). One nonclassical form of PR has been characterized in the cytoplasmic membranes of human sperm (3). The action of these R apparently involves opening of calcium channels in the mitochondrial and cellular membranes (5–7).

The expression of the two forms of PR, *i.e.* the nuclear and nonclassical forms, has been demonstrated in the ovary, particularly in granulosa cells (8–13). In the ovary, the level of PR expression is under hormonal regulation throughout the menstrual cycle by steroids and LH (8, 13–16). This R is suggested to play a key role in follicular development and maintenance of the corpus luteum (17–19). On the other hand, a recent study demonstrated the expression of an unusual PR in porcine granulosa cells, which mediates its actions through mobilization of Ca^{2+} from the endoplasmic reticulum through activation of phospholipase C (20). Adult female mice with disrupted classical PR displayed significant defects in reproductive functions, including anovulation and uterine hyperplasia and inflammation (21). In contrast, adult mutant male mice showed no impairment of fertility, indicating that, unlike in the female, nuclear PR is not essential for male reproduction. Studies of possible PR expression in the testis are scanty (22, 23), and there are no detailed studies on putative effects of PR on Leydig cell function. Only one report exists demonstrating P binding to the cytosol of rat Leydig cells (24).

Recently, we demonstrated the inhibitory effect of high P levels on LH receptor (LHR)expression and function in Leydig cells (25). The aim of the present study was to study further the mechanisms of the documented P actions on Leydig cells and to explore whether evidence could be obtained for the presence of classical or nonclassical PR to explain the functional responses detected. In this report we demonstrate the presence and function of a R in murine Leydig tumor cell line (mLTC-1) (26), which is distinct from the classical nuclear PR.

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Materials and Methods

Animals

Mouse tissues were obtained from adult NMR-1 mice kept under standard conditions and diet. The studies were approved by the Turku University committee of ethics for animal experimentation.

Ligand binding assays

The mLTC-1 cells were grown in HEPES-buffered Waymouth's medium (Life Technologies, Inc., Paisley, Scotland), as previously described (27). For the binding experiments, 7×10^4 cells/well were plated on 24-well culture dishes (Greiner, Frickenhausen, Germany), and 24 h later the binding experiments were performed on the living cells. Cells were incubated for 1 h at 37 C in serum-free Waymouth's medium with 2.2 nmol/liter [1,2,6,7-^{3}H]progesterone ([1,2,6,7-^{3}H]P; 4.40 tetrabecquerels/mmol; Amersham Pharmacia Biotech, Aylesbury, UK) in the presence or absence of 0.1–10 μmol/liter of one of the following nonlabeled steroids: P, cortisol, testosterone, estradiol, 17α-hydroxy-P, dehydroepiandrosterone (DHEA), pregnanediol (5β-pregnane-3α, 20α-diol), pregnanolone (5β-pregnen-3α-ol-20-one; all from Sigma, St. Louis, MO), or antiprogestin RU 486 (RU 38484, Roussel UCLAF, France).

For Scatchard analysis, the binding experiments were performed in the presence of 86 μmol/liter aminoglutethimide (AMG; Sigma) to block the endogenous steroidogenesis and in the presence or absence of excess cold P (50 μmol/liter) with increasing concentrations of [^{3}H]P (up to 30 nmol/liter). Cells were preincubated for 2 h with 86 μmol/liter AMG, and then the incubations for P binding were carried out in the presence or absence of 1 μmol/liter cortisol, to reduce nonspecific binding, as endogenous production of P was blocked by AMG. After a 1-h incubation with [^{3}H]P, the media were removed, and cells were washed with PBS, pH 7.4 (Life Technologies, Inc.) and lysed in 1% SDS containing 0.3 N NaOH solution. The lysates were transferred into scintillation vials, and the radioactivity was measured in a β-counter after addition of 1.5 ml scintillation liquid (Optiphase, Wallac, Inc. OY, Turku, Finland).

Isolation of RNA

The single step method (28) was used to isolate total RNA from control and treated samples for Northern hybridization analysis. Cells were stimulated in six-well culture dishes for 6 h in the presence or absence of 50 μg/liter hCG (CR-121; 13,500 IU/mg by bioassay; NICHHD, Bethesda, MD) and with or without 86 μmol/liter AMG or 10 μmol/liter of the selected steroids (see below). The tissues (testes, uterus, and spleen) were snap-frozen in liquid N_2 and stored at -70 C until isolation of RNA, and RT-PCR was carried out using RNA of high purity (29).

RT-PCR and Southern hybridization

The oligonucleotide primers used were designed according to the published complementary DNA (cDNA) sequence of the murine classical nuclear PR (30). The sense primer, 5′-CTAAATGAGCAGAGG ATGAAGGAG-3′, corresponding to nucleotides 2320–2343, and the antisense primer, 5′-TGGGGCAACTGGGGCAGCAATAAC-3′, corresponding to nucleotides 2725–2704, were used to amplify a 406-bp DNA fragment encoding the conserved steroid-binding domain of the classical PR. The RT and PCR reactions were performed sequentially in the same assay tube. Three micrograms of total RNA were used for all samples except the uterus, where 0.3 μg was used. RNA was reverse transcribed using the avian myeloblastosis RT (Finnzymes Oy, Espoo, Finland) and the above oligonucleotides as primers. The cDNAs generated were then amplified by PCR using the same primers as those described above in a mixture containing 1 nmol/liter of each primer, 200 μmol/liter deoxy-NTPs, 20 U ribonuclease inhibitor (RNasin, Promega Corp., Madison, WI), 12.5 U avian myeloblastosis RT, and 2.5 U *Taq* DNA polymerase. The reaction was started at 50 C for 10 min, followed by 3 min at 97 C, and then ran for 39 PCR cycles (97 C for 1 min, 55 C for 2 min, 72 C for 2 min, and final extension for 5 min at 72 C). An aliquot of the PCR products was analyzed on gel electrophoresis, and molecular sizes of the amplified products were determined by mol wt markers run in parallel with RT-PCR products.

The cDNA fragments generated from RT-PCR were resolved in 1.2% agarose gel and transferred onto nylon membranes (Hybond, Amersham Pharmacia Biotech, Arlington Heights, IL). The membranes were prehybridized for 4 h in 5 × SSP (0.18 mol/liter NaCl, 0.01 mol/liter sodium phosphate, and 1 mmol/liter EDTA, pH 7.7), 5 × Denhardt's solution [0.02% BSA, 0.02% (wt/vol) Ficoll, and 0.2% (wt/vol) polyvinylpyrrolidone], 0.5% SDS, and heat-denatured sonicated calf thymus DNA (20 mg/liter). The hybridizations were performed overnight at 42 C using a ^{32}P end-labeled nested oligonucleotide probe, 5′-TCGA-CAGCTTGCATGATCTTG-3′, corresponding to bases 2601–2621 of the classical PR cDNA. The membranes were washed twice for 10 min each time with 2 × SSPE-0.1% SDS at room temperature and then exposed to Kodak x-ray films (Kodak XAR-5, Eastman Kodak Co., Rochester, NY) for 4 days at -50 C. The molecular sizes of the RT-PCR amplicons were determined by comparison with molecular size markers.

Assessment of the RT-PCR products and Northern hybridization

The PR fragment produced by RT-PCR from adult mouse uterus was eluted from the agarose gel, and its identity was confirmed with the *Eco*RI restriction endonuclease digestion, which provided 100- and 306-bp fragments. The 406 PCR fragment was then directly cloned into a plasmid vector (T-vector, Promega Corp.), and its identity corresponded to the classical mouse PR sequence (30). T7 polymerase was used to generate a ^{32}P-labeled antisense complementary RNA (cRNA) probe using the cloned cDNA as template. Prehybridization and hybridization were carried out as previously described (31), and the membranes were washed in decreasing concentrations of SSC containing 0.1% SDS at 64 C and treated for 5 min at room temperature with 2 × SSC (1 × SSC = 0.15 mol/liter NaCl and 15 mmol/liter sodium citrate, pH 7.0) in the presence of ribonuclease-A (2 mg/liter), followed by two washes in 0.1 × SSC and 0.1% SDS at 64 C. The membranes were exposed to Kodak x-ray films for 5 days at -50 C before and after the ribonuclease treatment.

Western and ligand blot analysis

The mLTC-1 cells from six-well culture plates were scraped off into PBS under ice, washed, and lysed in 50 μl buffer [20 mmol/liter Tris (pH 7.4), 150 mmol/liter NaCl, 0.25% Nonidet P-40, 1 mmol/liter Na_3VO_4, and 1 mmol/liter PMSF]. The proteins were measured in the cell lysates using a Bio-Rad Laboratories, Inc., kit (Hercules, CA), and 50 μg protein were diluted in an equal volume of 2 × Laemmli's reducing sample buffer. The samples were vortexed, incubated at 95 C for 5 min, and then loaded onto 10% polyacrylamide-bisacrylamide midigels. After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes (Sigma). The lysates of human sperm from healthy donors were used as controls. The transferred nitrocellulose membranes were blocked for 3 h at room temperature in TTBS (0.1% Tween-20, 20 mmol/liter Tris, and 150 mmol/liter NaCl) containing 5% BSA. Thereafter, the membranes were washed repeatedly in TTBS and incubated for 2 h in 2% BSA-TTBS containing a 1:400 dilution of the αc-262 PR antibody directed against the C-terminal region of the classical PR (32). Another antibody (α-PR, at a 1:100 dilution) was also used, which recognizes the N-terminal region of the classical PR. After washing, the membranes were incubated with goat antimouse IgG-POD (1:5000 in 2% BSA-TTBS). The membranes were then washed several times in TTBS, and immunoreactive proteins were visualized by the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). Alternatively, for the ligand blot analysis, the nitrocellulose membranes were incubated for 30 min in 3% Nonidet P-40/0.1% BSA, then for 2 h in 0.3% BSA/0.1% Tween-20/PBS, followed by 10 min in 0.1% Tween-20/PBS, and finally overnight in 0.3% BSA/0.1% Tween-20/PBS with or without a 10-fold concentration of P containing 1 μmol/liter peroxidase-conjugated P (P-POD; Sigma). Peroxidase was conjugated to P by carboxymethyl oxime at position 3 [progesterone 3-(*O*-carboxymethyl)oxime-6-amino caproic acid-POD type IV]. After several washes in 0.1% Tween-20/PBS, the protein bands binding P were visualized by ECL Western blotting kit.

Intracellular free Ca^{2+} measurements

To test the P actions on intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentrations, mLTC-1 cells were plated on glass coverslips at a density of 3 ×

10^4 cells/cm^2. Cells were loaded with 2 μmol/liter fura-2/AM (Sigma) at 37 C for 30 min in serum-free culture medium. The cells were incubated further for 10 min with serum-free medium to wash off the unincorporated fura-2/AM. The coverslips were taken into assay buffer (137 mmol/liter NaCl, 5 mmol/liter KCl, 0.44 mmol/liter KH_2PO_4, 4.2 mmol/liter $NaHCO_3$, 1.2 mmol/liter $MgCl_2$, 20 mmol/liter HEPES, 1 mmol/liter $CaCl_2$, and 10 mmol/liter glucose, pH 7.0) on ice until $[Ca^{2+}]_i$ measurements were performed using the Hitachi F-2000 double wavelength fluorometer (Hitachi, San Jose, CA). Calibration for the measurements was performed using 0.2% (wt/vol) digitonin (Sigma) and 3 μmol/liter EGTA. The concentration of P (1 μmol/liter) was used for ($[Ca^{2+}]_i$) determination. Alternatively, mLTC-1 cells were trypsinized, centrifuged, and loaded with fura-2/AM at 37 C for 30 min, followed by ($[Ca^{2+}]_i$) measurement in the suspension, as described above.

The immediate action of P on the Ca^{2+} entry was studied in mLTC-1 cells plated on 24-well dishes at a density of 6×10^4 cells/well. Twenty-four hours later, cells were washed once with Ca^{2+}-free medium (Life Technologies, Inc.), and 500 μl Ca^{2+}-free medium containing 5 μCi/ml $^{45}Ca^{2+}$ isotope (Ca^{45}, NEN Life Science Products, Boston, MA) were added to each well in the presence or absence of varied doses of P (0.1–10 μmol/liter). The mLTC-1 cells were incubated for 15 min at 37 C, washed, and lysed in 500 μl 1% SDS containing 0.3 N NaOH. The lysates were counted in a β-spectrometer (Rack β, Wallac, Inc., Turku, Finland) after adding 1 ml scintillation liquid.

Steroid regulation of LHR messenger RNA (mRNA) expression

The mLTC-1 cells (7×10^5/well in six-well plates) were cultured for 6 h in the presence or absence of 10 μmol/liter P, cortisol, testosterone, estradiol, 17-hydroxyprogesterone (17-OHP), DHEA, pregnanediol, pregnanolone, or the antiprogestin RU 486. In additional experiments, cells were also treated for 48 h with increasing concentrations of P (0.1–10 μmol/liter). Fifteen to 20 μg total RNA were analyzed by Northern hybridization using an antisense ^{32}P-labeled cRNA probe corresponding to bases 441–849 of extracellular domain of the rat LHR cDNA (33). The hybridized membranes were exposed to x-ray films (Kodak) for 5 days at −70 C. The intensities of the specific mRNA species were quantified using the Tina software (Raytest, Straubenhardt, Germany) and normalized according to the intensities of the 18S ribosomal RNA bands in the gels stained with ethidium bromide.

[^{125}I]Iodo-hCG binding

Radioiodination of hCG (CR-121, NIDDK) was carried out with Na[^{125}I]iodide (IMS 300, Amersham Pharmacia Biotech), using a solid phase lactoperoxidase method (34). The specific activity of the labeled hormone was 30 μCi/μg, and hCG binding studies were conducted under optimized conditions, as described previously (25). Briefly, mLTC-1 cells were treated for 48 h in the presence or absence of 10 μmol/liter of one of the steroids, and 3×10^5 cells were incubated with [^{125}I]iodo-hCG (~10^5 cpm/incubation) in the absence (total) or presence (nonspecific) of 50 IU unlabeled hCG (Pregnyl, Organon, Oss, The Netherlands). The reaction was terminated after overnight incubation in 3 ml ice-cold Dulbecco's-PBS containing 0.1% BSA. After centrifugation, supernatant was discarded by careful aspiration, and pellet was counted in a γ-spectrometer (1260 Multigamma II, Wallac, Inc.).

Statistics

All results presented are from two to four independent experiments (unless otherwise specified). The data were analyzed by one-way ANOVA, followed by Duncan's new multiple range test, and $P < 0.05$ was considered statistically significant. The results shown in the figures represent the mean ± SEM.

Results

P binding studies

The Scatchard analysis (Fig. 1A) revealed that cultured mLTC-1 cells bound [^{3}H]P with a high and a low affinity component, the former having a K_d of 9.3 ± 4.6 nmol/liter and the latter having a K_d of 284 ± 12.8 nmol/liter, in the presence of 1 μmol/liter cortisol, to exclude the confounding effect of glucocorticoid receptors. Determinations of the affinities of P in the absence of cortisol or binding carried out in ice provided similar results (data not shown). In ligand displacement assays, the [^{3}H]P binding was displaced by increasing concentrations of P, 17-OHP, and DHEA in the incubation medium. Other steroids studied, including the antiprogestin RU 486, cortisol, and testosterone, failed to displace P binding (Fig. 1, B and C). On the other hand, a 2-h incubation with AMG increased the specific P binding of mLTC-1 cells (Fig. 1D).

PR mRNA detection by RT-PCR and Southern hybridization

The results of RT-PCR analysis revealed a cDNA amplicon of approximately 400 bp size in mouse uterus, testis, and mLTC-1 cells, but not in mouse spleen (Fig. 2). However, the cDNA fragment from the uterine RNA was the only one that was confirmed by restriction endonuclease digestion using *Eco*RI to be cleaved into the expected 100- and 300-bp fragments (data not shown). In accordance, Southern hybridization using a ^{32}P end-labeled nested oligonucleotide probe revealed strong positive hybridization to the uterine sample and weak signal to the testis, but no hybridization was found to the spleen or mLTC-1 cell lysates. The RT-PCR products of testis and mLTC-1 cells were subcloned into the T-vector (Promega Corp.), and their sequencing revealed homology to phospholipase A2 (result not shown). In contrast, restriction endonuclease digestion and sequencing of the uterine amplicons revealed identity with the classical murine PR (30), and the cDNA was later used as a template for PR cRNA in Northern hybridization (see below).

Northern hybridization for the classical nuclear PR

To confirm that the classical PR is not expressed in the mLTC-1 cells, we performed Northern hybridization analysis using the cDNA obtained from the RT-PCR amplification of the uterus samples as template after confirming its sequence identity. The mLTC-1 cells were treated for 6 h in the presence or absence of 86 μmol/liter AMG or/and 50 μg/liter hCG with or without 5 μmol/liter P to investigate the possible regulatory pattern of the nuclear PR after different hormonal stimulations. However, in agreement with the RT-PCR data, no specific PR mRNA signal was observed in these cells, with or without hormonal treatment (data not shown), or in mouse spleen; only the adult mouse uterus RNA, used as a positive control, displayed a clear hybridization signal (Fig. 3).

Identification of P-binding proteins in mLTC-1 cells

As mLTC-1 cells did not express the classical PR gene at the mRNA levels, our hypothesis was that these cells might possess a PR form immunologically related to that recently discovered in human sperm (6). The amino acid structure of the C-terminal region of the classical PR is supposed to be conserved also in the sperm membrane PR (32). Therefore, we compared the PR immunoreactivity in mLTC-1 cells and

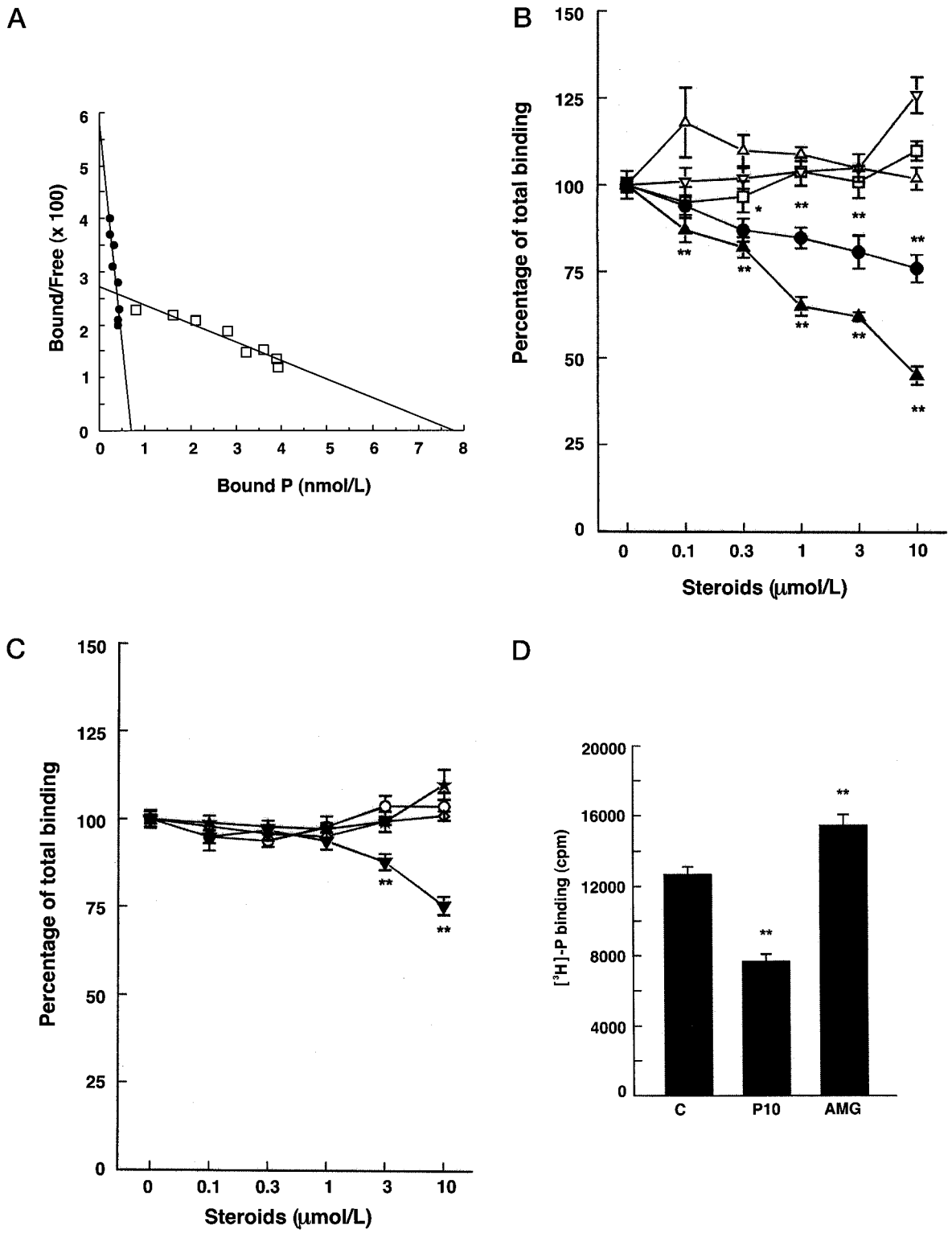

FIG. 1. Scatchard analysis of P binding to the mLTC-1 cells (A) and P ligand displacement assays (B-D). For the Scatchard analysis, a constant number of cells were incubated in the presence of increasing concentrations (1–30 nmol/liter) of [^{3}H]P together with constant amount of excess cold P (50 μmol/liter) to determine nonspecific binding in the presence of 1 μmol/liter cortisol. In B and C, the mLTC-1 cells (7×10^4/well) were incubated with 2.2 nmol/liter [^{3}H]P in the absence and presence of increasing concentrations (0.1–10 μmol/liter) of P (*solid triangles*), 17-OHP (*solid inverted triangles*), DHEA (*solid circles*), cortisol (*open squares*), estradiol (*open triangles*), RU 486 (*open inverted triangles*), testosterone (*open circles*), pregnanolone (*open diamonds*), or pregnanediol (*solid stars*). The results are presented as a percentage of total binding in the absence of cold ligand. The [^{3}H]P binding to mLTC-1 cells is shown in D after a 2-h incubation with 10 μmol/liter P (P10) or 86 μmol/liter AMG. C, Control. The results are the mean ± SEM of 8–20 replicates from 2–5 individual experiments. *, $P < 0.05$; **, $P < 0.01$.

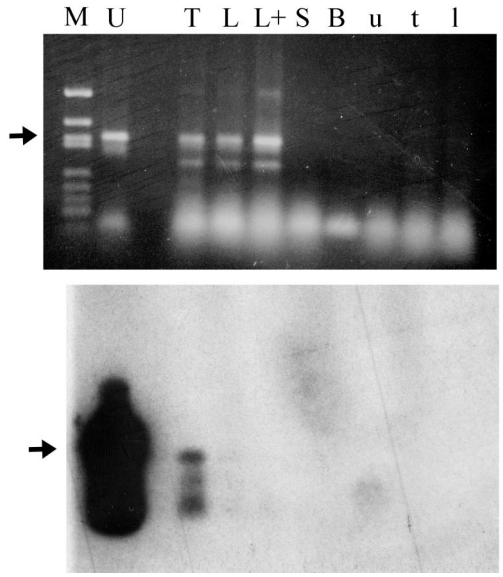

FIG. 2. RT-PCR amplification (*upper panel*), followed by Southern hybridization (*lower panel*), of nuclear PR mRNA in adult mouse uterus (U), testis (T), spleen (S), or mLTC-1 cells in the presence (L+) or absence (L) of 50 μmol/liter 8-bromo-cAMP for 6 h before RNA isolation. The RT-PCR reaction was also performed on a buffer-only sample (B), and by omitting reverse transcriptase from the reactions (*small letters*). M, Mol wt markers. The *upper panel* shows the gel stained with ethidium bromide, and the *lower panel* shows the Southern hybridization x-ray image using a nested oligonucleotide probe for the samples of the *upper panel* after transfer onto a Nylon membrane. The *arrow* shows the mobility of a cDNA band of the expected 400 bp size.

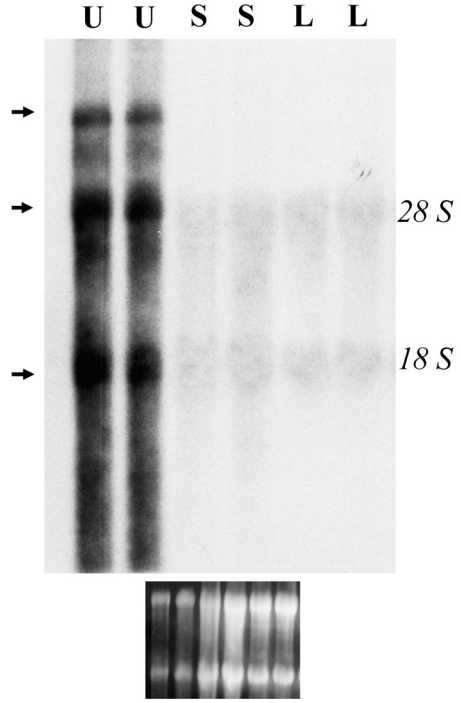

FIG. 3. Northern hybridization of the nuclear PR mRNA in samples from adult mouse uterus (U), spleen (S), and mLTC-1 cells (L). The *arrows* on the *left* indicate the expected sizes of the PR transcripts in the uterus (7.5, 4.4, and 2.4 kb); migration of the 18S and 28S ribosomal RNAs is shown on the *right*. The *lower panel* shows ethidium bromide staining of the gel as indicator of RNA loading.

human sperm lysates by Western blot analysis using an antibody (αc-262) known to recognize the C-terminal region of the classical nuclear PR. This antibody is known to recognize all species of PR, including the cell membrane PR in human sperm (35).

In sperm lysates, we observed several immunoreactive bands between 45 and 66 kDa, and proteins with 54 and 66 kDa sizes gave the strongest signals. We also tested another antibody (α-PR), which is directed against the N-terminal domain of the classical PR. This antibody has recently been shown to recognize a 66-kDa protein in human sperm lysates (35). However, we observed that the αc-262 antibody detected in mLTC-1 cell lysates similar immunoreactive protein bands between 45 and 57 kDa, whereas the α-PR antibody faintly recognized proteins of the same sizes (Fig. 4A). In addition, both antibodies identified two additional bands of similar intensities at 35 kDa, which were absent in sperm lysates and may be nonspecific. Importantly, using either antibody, the mLTC-1 cells did not clearly show the 66-kDa band, but had similar immunoreactive proteins at 45–57 kDa (Fig. 4A). Ligand blot analysis was then performed using P-POD as probe to reveal which proteins in the lysates can specifically bind to P. In both sperm and mLTC-1 cell lysates, P-POD bound largely to the same proteins revealed by the αc-262 and α-PR antibodies, and most of the bands were displaced with a 10-fold excess of free P, except for a 50-kDa band in the sperm lysates (Fig. 4B).

P regulation of intracellular free $Ca^{2+[I]}$ in mLTC-1 cells

A P concentration of 1 μmol/liter induced an immediate increase in the ($[Ca^{2+}]_i$) levels in mLTC-1 cells cultured on glass chips and loaded with fura-2/AM. The effect was comparable with the maximal signal obtained after permeabilization of the cell membranes by digitonin (Fig. 5A). In addition, 0.1–3 μmol/liter P increased $^{45}Ca^{2+}$ entry into mLTC-1 cells after a 15-min incubation. The $^{45}Ca^{2+}$ entry returned to normal levels at the high (10 μmol/liter) concentrations of P (Fig. 5B).

Steroid regulation of LHR expression

We next examined the specificity of P action on the steady state levels of LHR mRNA. After a 6-h treatment with 10 μmol/liter P or cortisol, we observed down-regulation of LHR mRNA expression (Fig. 6). In addition, DHEA and 17-OHP displayed moderate, but significant, inhibition of the LHR mRNA signal. The effect was confined to these steroids, as no effects were found after 6-h treatment with the other steroids tested (Fig. 6), including the antiprogestin RU 486. The effects of the selected steroids on [^{125}I]iodo-hCG binding to the mLTC-1 cells were qualitatively similar after 48-h treatment at 10 μmol/liter concentrations of the steroids (Fig. 7).

To further ascertain the observed inhibitory effect of P on LHR expression, we next measured the effect of increasing concentrations of P (0.1–10 μmol/liter) on LHR mRNA levels. The results presented in Fig. 8 show dose-dependent inhibition of LHR mRNA content with increasing P concen-

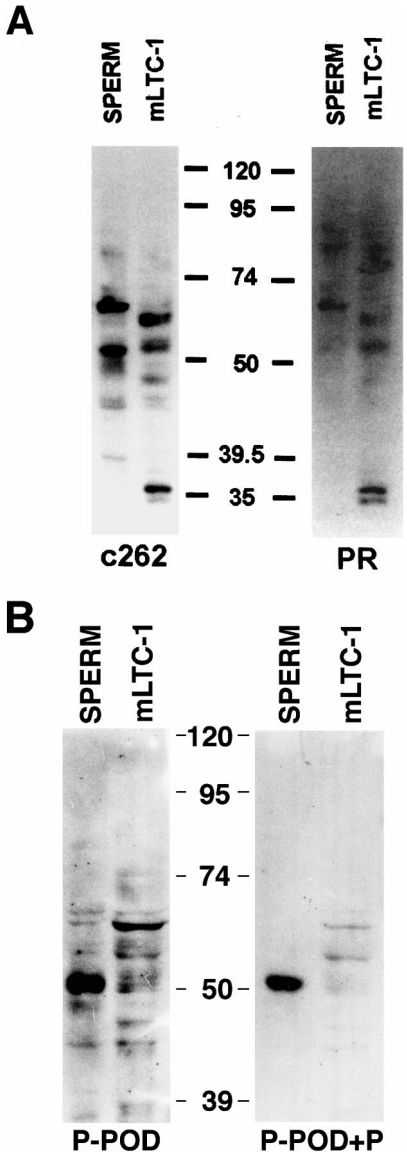

FIG. 4. Western hybridization (A) and ligand blot assays (B) for PR in protein lysates from human sperm and mLTC-1 cells. The immunoreactive proteins were detected after transfer onto cellulose membranes, using αc-262 (c262) or α-PR (PR) antibody. B shows the proteins binding to P-POD in the absence or presence (+P) of a 10-fold excess of P (10 μmol/liter).

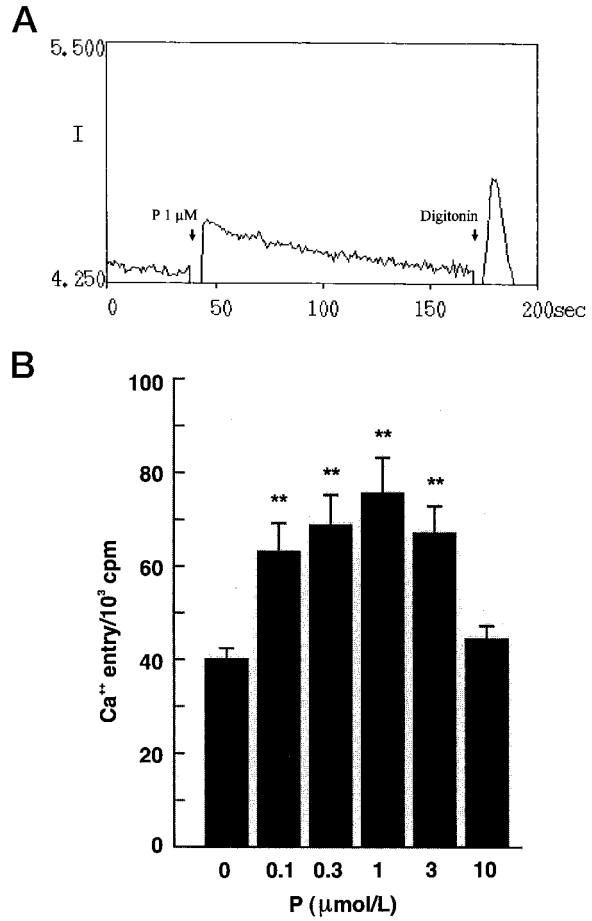

FIG. 5. Measurement of intracellular free Ca^{2+} using the fura-2/AM method in mLTC-1 cells cultured on coverslips (A). Cells were stimulated with 1 μmol/liter P or with 0.2% digitonin (to yield the maximum response). The graph presents one of three independent experiments with similar results. B shows $^{45}Ca^{2+}$ entry into mLTC-1 cells on 24-well plates after 15-min incubation with 0.1–10 μmol/liter P. Each *point* represents the mean ± SEM of eight replicates from two independent experiments. **, $P < 0.01$ *vs.* control.

trations. These results further confirmed the previous data regarding involvement of P in the down-regulation of LHR mRNA.

Discussion

Expression of the classical nuclear PR mRNA was not observed in mLTC-1 cells, even using the sensitive RT-PCR method. The adult mouse testis, however, showed weak expression of the nuclear PR mRNA, and it might be expressed in other testicular cell types than the Leydig cells.

The results of Western blot demonstrated several PR-like immunoreactive proteins between 45–57 kDa in the mLTC-1 cell lysates. These bands were especially observed when an antibody raised against the steroid-binding domain of the classical nuclear PR (αc-262) was used. This antibody is able to detect the classical PR in breast cancer cells (32), human uterus (36), and insect cells transfected with the A form of PR (35); however, the sizes of those reactive bands are 120, 94, and 60 kDa (35, 36). The mouse uterus PR species are also similar in size, ranging between 66–115 kDa (30). The αc-262 has been shown to prevent P-mediated calcium influx into human sperm when added to the incubation medium (36). Using this antibody we were able to show that mLTC-1 cells only expressed shorter forms of PR that were close in size to those observed in human sperm lysates (36).

The immunoreactive P-binding proteins in sperm and mLTC-1 cell lysates were also faintly detected by another antibody (α-PR) that is directed toward the N-terminal domain of the classical nuclear PR. They apparently represented minimal cross-reactivity and suggested the specificity toward the C-terminal region. These findings together with the fact that no expression of the nuclear PR mRNA was observed in mLTC-1 cells indicated that these cells may express an unusual form of PR. Moreover, the pattern of steroid

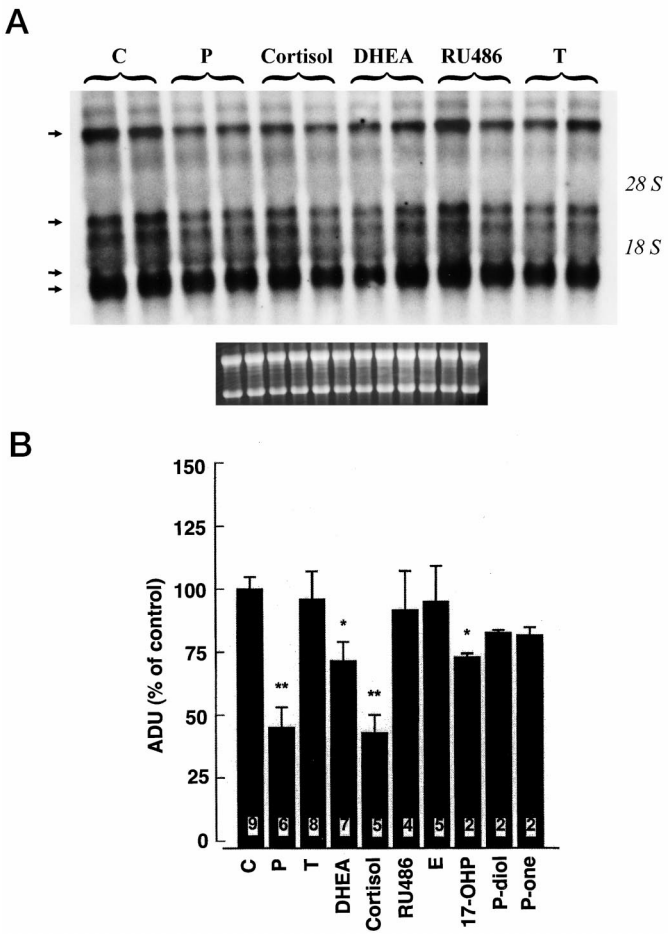

FIG. 6. Northern hybridization analysis of the effects of selected steroids on LHR mRNA in mLTC-1 cells. Cells were cultured for 6 h in the presence or absence of 10 μmol/liter P, cortisol, DHEA, RU 486, testosterone (T), estradiol (E), 17-OHP, pregnanolone (P-one), or pregnanediol (P-diol; the last four steroids are not shown in the x-ray image). The *upper panel* shows an example of the Northern hybridizations. A specific cRNA probe for the extracellular part of the LHR (nucleotides 441–849) was used. The locations of the 28S and 18S ribosomal RNAs are indicated on the *right*. The *arrows* on the *left* indicate the positions of the different LHR mRNA splice variants (7.7, 4.0, 2.7, and 1.8 kb) on the x-ray films. *Below* the x-ray image, the ribosomal RNA bands are shown in the gel stained with ethidium bromide. The *lower panel* shows the densitometric quantification of the longest (~7.7 kb) LHR mRNA transcript related to the density of the 18S ribosomal RNA band from the ethidium bromide-stained image. Each *bar* represents the mean ± SEM from one to four independent experiments, and the number of replicates is indicated at the *bottom* of each bar. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control).

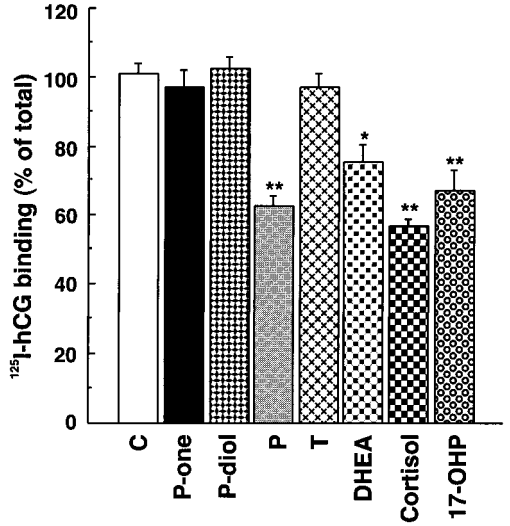

FIG. 7. Effects of different steroid hormones on [^{125}I]iodo-hCG binding. The mLTC-1 cells were stimulated for 48 h in the absence (C) or presence of 10 μmol/liter of each hormone, and [^{125}I]iodo-hCG binding was assessed with fixed amount of labeled hCG (~10^5 cpm/tube), as described in *Materials and Methods*. The results are the mean ± SEM of triplicate determinations from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control).

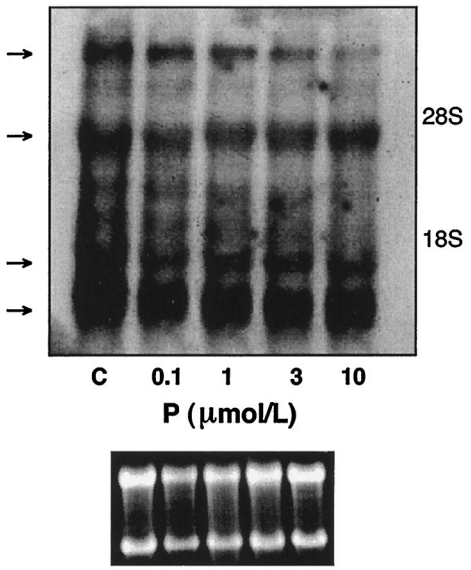

FIG. 8. Dose response of the P effect on LHR mRNA expression in mLTC-1 cells by Northern blot analysis. Cells were treated without (C) or with increasing concentrations of P (0.1–10 μmol/liter). Total RNA was extracted from different treatment groups and subjected to determination of LHR mRNA levels, as described in *Materials and Methods* and Fig. 6. A representative autoradiogram is shown, and the approximate positions of the 18S and 28S ribosomal RNAs are indicated on the *right*, whereas the different LHR mRNA splice variants (7.7, 4.0, 2.7, and 1.8 kb) are shown by *arrows* on the *left* (*upper panel*). The ethidium bromide staining of ribosomal RNAs demonstrate equal RNA loading (*lower panel*). Similar results were obtained from three independent experiments.

binding suggested different ligand specificity for the R. Interestingly, a recent study (37) demonstrated that P binds to the specific proteins purified from porcine liver with two binding affinities (K_d, 11 and 286 nmol/liter). The Scatchard analyses of P binding in the mLTC-1 cells appear to have two different affinity components at similar ranges of K_d values, *i.e.* 9.3 ± 4.6 and 284 ± 12.8 nmol/liter. In ligand blot assays, only some bands completely disappeared in the presence of a 10-fold excess of cold P, whereas others were only decreased in intensity. Although the antibody used did not clearly recognize the 60-kDa band in the mLTC-1 cell lysate, a protein of similar size was revealed using P-POD as the detection system. It is suggested that some forms of PR in mLTC-1 cells are similar, yet not identical, to the sperm membrane PR, which may be due to differences in the structure and function of the two cell types, as spermatozoa are

terminally differentiated cells whereas mLTC-1 cells are immortalized Leydig tumor cells. Subsequently, Western and ligand blot analyses also confirmed the absence of classical nuclear PR in mLTC-1 cells. As the gene of the nonclassical sperm PR is not yet known, we are currently unable to study whether the apparent differences in the sperm and mLTC-1 cell PR-representing molecules are products of different genes or due to alternative splicing or posttranslational modification of the same gene.

Similar to its reported actions in granulosa cells (20) and human sperm (3), P also induced a rapid increase in $[Ca^{2+}]_i$ in mLTC-1 cells. Nonspecific interaction of P in increasing $[Ca^{2+}]_i$ can be ruled out, as the ethanol concentration (0.01%) used for P dilution was without effect (data not shown). Ca^{2+} is a known regulator of many enzymes, including protein kinase C (PKC), where it is known to participate in its translocation to the cytoplasmic leaflet of the cell membrane (38), to be activated there by diacylglycerol. The activated PKC, in turn, serves as an interacting signal in the regulation of gene expression. In accordance, the induction of PKC is known to down-regulate LHR expression and function in Leydig tumor cells (39). The classical pathway for the nuclear PR in the regulation of LHR expression is unlikely, as the murine LHR promoter does not contain a complete P response element within appropriate distance from the transcription initiation site (40). The P-mediated increase in $[Ca^{2+}]_i$ was observed in mLTC-1 cells cultured on coverslips, but not in trypsinized cells. This may point out that the observed P effect is mediated through a plasma membrane PR, which is disturbed after trypsinization of these cells or that other signaling pathways important for Ca^{2+} immobilization/influx are disturbed after this procedure. In the ovary, PR is down-regulated by elevated concentrations of P or its metabolites (41). The decrease in P binding and action upon Ca^{2+} entry, when measured in cells plated for 48 h, might be due to down-regulation by the accumulated high basal production of P or its metabolites.

The affinity of the mLTC-1 cell PR for its ligand may be different in cytosolic extract and whole cell preparation. The P actions on Leydig cells require high, *i.e.* micromolar, concentrations of this steroid, also indicating that mouse Leydig tumor cells express a special form of PR. This is logical in view of the fact that PR in Leydig cells needs elevated ligand concentrations to reach the activation threshold. The P concentration in the testis in basal *in vivo* conditions is far higher than that in the blood, and it reaches up to 0.3–3 μmol/liter in testes when adult rats are treated with a high (600 IU/kg) down-regulating dose of hCG (42). Therefore, despite the apparently high levels of P that are needed to elicit an effect on Leydig cells, this regulatory system can well be physiological, acting at the site of P synthesis, where very high hormone levels are attained. In this respect, the nonclassical PR in mLTC-1 cells is relatively similar to the receptor characterized in human sperm, as in both cases, P acutely increases the Ca^{2+} flux. Noteworthy, nonclassical binding sites for P have been demonstrated in bovine follicular and luteal membranes, whether they have been suggested to be involved in autocrine/paracrine regulation of follicular function (43, 44). We recently showed that P regulates LHR function in primary cultures of adult mouse Leydig cells (25). In addition, involvement of P inhibits LHR mRNA levels in mLTC-1 cells in a dose-response manner. Thus, our present results may not only be confined to a specific tumor cell, but also to normal Leydig cells. We also investigated the effects of selected steroids in the regulation of LHR function in mLTC-1 cells by quantitative [^{125}I]iodo-hCG binding studies, and the pattern of effects was similar to their actions on LHR mRNA in primary adult Leydig cells (El-Hefnawy, T., unpublished observations).

Although cortisol displayed an inhibitory effect on LHR mRNA and binding [in agreement with our recent report (25)], the competition assay for P binding to mLTC-1 cells showed that cortisol does not compete with P for binding to the same R. As the glucocorticoid receptor is expressed in Leydig cells (45–47), the cortisol effects on cultured Leydig cells are apparently mediated through its own receptor and/or by an pathway independent from that of P action. Another difference between the two steroids is that corticosterone affects testicular function in an endocrine fashion originating from the adrenal gland, whereas the effect of P on Leydig cells seems to be of an autocrine nature.

In conclusion, our study sheds more light on direct P effects on Leydig tumor cells. The P binding detected in Leydig cells is different from the classical nuclear PR, but probably structurally and functionally related to the membrane variant of PR that has recently been characterized in human sperm. The signaling pathway of this R involves increased $[Ca^{2+}]_i$ levels, through which P may regulate LHR gene regulation in Leydig cells, possibly through PKC and other Ca^{2+}-dependent pathways. Further studies of the physiological significance will clarify the novel P actions as well as other signaling pathways involved in Leydig cells.

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