Establishment of Gonadotropin-responsive Murine Leydig Tumor Cell Line

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ABSTRACT Several clonal Leydig tumor cell lines have been established by adapting the transplantable Leydig tumor, M548OP, to culture. One of these cell lines, MLTC-1, has been characterized with regard to the gonadotropin-responsive adenylate cyclase system. The binding of ^{125}I-labeled human chorionic gonadotropin (hCG) was blocked by excess unlabeled hCG and lutropin (LH) but not by follitropin, thyrotropin, or insulin, indicating the presence of specific receptors for hCG and LH. Based on the specific binding of hCG to isolated MLTC-1 membranes, the calculated dissociation constant was $1.0 \pm 0.2 \times 10^{-10}$ M. The receptors appeared identical to those from normal murine Leydig cells when analyzed by SDS PAGE and sucrose density gradient centrifugation. The molecular weight and sedimentation coefficient were 95,000 daltons and 8.5 S, respectively. MLTC-1 cells responded to hCG by accumulating cyclic AMP and producing progesterone. Cyclic AMP accumulation was time- and dose-dependent with a maximal accumulation occurring at ~0.2 nM hCG. At saturating levels of hCG, cAMP levels reached a maximum by 30 min and then declined very slowly. Adenylate cyclase activity in membranes prepared from MLTC-1 cells was stimulated by hCG, LH, NaF, cholera toxin, and guanyl-5'-ylimidodiphosphate. Additionally, choleragen was found to ADP-ribosylate a membrane protein of 54,000 daltons. This protein resembles the proposed guanine nucleotide regulatory component in both size and choleragen-dependent reactivity. These data suggest that MLTC-1 cells possess a gonadotropin-responsive adenylate cyclase system consisting of a specific hormone receptor, a regulatory component, and a catalytic subunit.

A number of polypeptide hormones mediate their effects on target cells by binding to specific cell surface receptors and activating adenylate cyclase (1, 2). This process has been studied in many cells, and investigations have been facilitated by the use of cultured cell lines. There has been, however, a lack of any cell line capable of responding to lutropin (LH) or its analog, human chorionic gonadotropin (hCG), until the recent independent development of clonal Leydig tumor cell lines in this laboratory (3) and in that of Ascoli (4, 5). These cell lines were derived from the transplantable murine Leydig tumor designated as M548OP (6). Work in this laboratory has produced nine clonal cell lines that bind hormone and demonstrate increased adenosine-3',5'-monophosphate (cAMP) production as a result. The purpose in developing such cell lines is to study the chain of events initiated by the binding of hCG to these cells and to increase our understanding of the components instrumental in executing these events. In this report I describe some properties of one of the nine Leydig tumor cell lines (MLTC-1) as it relates to the gonadotropin-responsive adenylate cyclase system. The principal emphasis is to demonstrate that MLTC-1 contains the essential components of this hormone-stimulated cyclase system and that the hormone receptor from MLTC-1 behaves the same as that from normal murine Leydig cells when the cross-linked hormone-receptor complex is analyzed by either SDS PAGE or sucrose density gradient centrifugation.

MATERIALS AND METHODS

Establishment of Clonal Cell Lines from the Leydig Cell Tumor M548OP

The M548OP Leydig tumor was initially obtained from the Papanicolaou Cancer Research Institute, Miami, FL. After several serial transplants in C57Bl/6J male mice, the tumor tissue was frozen in medium 199 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 20% fetal calf serum and 10% glycerol and maintained in liquid nitrogen. Immediately before injection,

it was thawed quickly in a 37°C water bath. Implantation of the tumor under semisterile conditions into anesthetized mice was achieved by subcutaneous injection of the tissue just posterior to the rib cage with a liver biopsy needle.

Cells from tumor-bearing mice were used to establish a cell culture system. The tumor was removed from the animal and cut into small pieces that were washed in several changes of sterile RPMI-1640 medium (M. A. Bioproducts, Walkersville, MD) and teased apart with forceps to liberate the cells. Cells were allowed to settle and attach themselves to the culture dish (25-cm^2 flasks; Falcon Plastics, Oxnard, CA). The medium was changed twice during the next several days. On the fourth day, the cells were transferred to a new flask after being detached by forceful pipetting. Henceforth, cells were grown in 25-cm^2 flasks using RPMI medium with 10% fetal calf serum (K. C. Biological, Lenexa, KS), 50 U of penicillin/ml, and 50 μg of streptomycin/ml under an atmosphere of 5% CO_2 in air at 37°C.

Subculturing was achieved by replacement of the medium with a minimal amount of 0.25% trypsin in Ca^{2+}/Mg^{2+} free Hanks' balanced salt solution containing 0.06% EDTA. The cells detached from the flask after ~10 min at 37°C and were diluted with medium and subdivided into new culture flasks. For the purpose of cloning, cells were diluted to an average density of two cells/ml, and 1-ml portions were dispensed into the wells of multiwell tissue culture dishes (Limbro Scientific, Hamden, CN). Colonies from wells containing only one cell initially were propagated as clonal cell lines. Once established, MLTC-1 was subcultured weekly at a ratio of 1:5.

Cross-linking and Analysis of Cross-linked hCG-receptor Complexes from MLTC-1 and Normal Testicular Membranes

Normal murine testicular membranes were isolated as described by Dufau et al. (7). Membranes from MLTC-1 were prepared by scraping cells from their culture flasks in PBS and homogenizing them with a Dounce homogenizer. The homogenate was centrifuged at 120 *g* for 20 min; the supernatant was decanted and centrifuged at 27,000 *g* for 30 min to yield a crude membrane pellet. These and all subsequent centrifugations were done at 4°C unless otherwise noted. The membranes were suspended in PBS and 0.5 to 1 mg of membrane protein was incubated in a final volume of 1 ml in the presence of 2×10^6 disintegrations/min (dpm) of ^{125}I-hCG (~5×10^{-10} M) and 0.1% bovine serum albumin (BSA) for 1 h at 37°C. At the end of the incubation, the samples were diluted with 3 ml of cold PBS and membranes were collected by centrifugation at 6,700 *g* for 15 min.

Membrane-bound hormone was cross-linked to its receptor using disuccinimidyl suberate (DSS) as described by Rebois et al. (8) and was analyzed either by SDS PAGE (8, 9) or by sucrose density gradient centrifugation (10). Samples of membrane containing 200–400 μg of protein were extracted with 1% Triton X-100 in 2 mM Tris-HCl, pH 7.4, for 30 min at room temperature. After adding marker proteins (^{14}C-labeled ovalbumin and globulins), the extracts were placed on 5-ml linear sucrose gradients (5%–20%) made up in 50 mM Tris-HCl, pH 7.4, with 0.1% Triton X-100 and centrifuged at 120,000 *g* for 16 h. Gradients were fractionated and ^{125}I and ^{14}C were assayed by gamma and liquid scintillation counting, respectively.

Electrophoretic analysis of the cross-linked hormone-receptor complex was carried out using a modification of the procedure described by Rebois et al. (8). After solubilization in 50 mM Tris-HCl, pH 6.7, 0.1% glycerol and 1% SDS for 30 min at 50°C, the sample containing 1–2 mg of membrane protein was centrifuged for 30 min at 100,000 *g* in a Beckman airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The resulting supernatant was passed over a 0.9 × 24 cm Sephadex G-200 column equilibrated with 50 mM Tris-HCl, pH 6.7, and 0.1% SDS. The fractions containing the hormone-receptor complex were combined and lyophilized before electrophoresis.

Hormone Binding to MLTC-1 Cells and Membranes

Binding of hormone to a clonal Leydig tumor cells was performed in culture flasks. The growth medium was replaced with 2 ml of fresh medium containing ^{125}I-hCG. Unlabeled hCG or other hormones were present in some experiments. After incubating for 1 h at 37°C, the medium was removed and the flasks were washed twice, each time with 2 ml of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) at 4°C. Cells were dissolved using 1 ml of 2 M NaOH, transferred to appropriate tubes with an additional 0.5-ml wash, and counted in a Beckman model 4000 gamma counter (Beckman Instruments, Inc.). Portions were removed for protein estimation.

Determination of the dissociation constant (K_d) for hCG was done with membranes isolated from a crude cell homogenate by centrifugation at 27,000 *g* for 30 min. The membranes were resuspended and 100-μl samples containing 200–300 μg of protein were incubated in a final volume of 0.4 ml with 0.1% BSA, 0.01% sodium azide, and various amounts of ^{125}I-hCG. Membranes were incubated for 14 h at 37°C before pelleting at room temperature with a Beckman microfuge (5 min at 10,000 *g*) (Beckman Instruments, Inc.). The pellets were washed once before measuring bound radioactivity. For estimating the number of binding sites, a crude homogenate was used without further purification. The incubation was shortened to 2 h and the components of the incubation mixture, exclusive of sodium azide, were the same as those described for the first method. Membranes were collected by a filtration technique described previously (11), except that Millipore type EG filters with a 0.2-μm pore size were used and the filters were washed with 0.2% BSA in PBS. Nonspecific binding was determined for both cells and membranes by adding 0.2 μM unlabeled hCG to the incubations.

Choleragen-induced ADP-Ribosylation

ADP-ribosylation was carried out on a crude membrane fraction isolated in the following manner. After removal of the growth medium, cells were scraped from flasks in a small amount of solution containing 1 mM EDTA, 0.2 mM dithiothreitol (DTT), and 2 mM Tris-HCl, pH 7.6 (12). The cells were then homogenized with a Dounce homogenizer. The homogenate was centrifuged at 400 *g* for 5 min and the resulting supernatant was centrifuged at 31,000 *g* for 20 min. The final pellet containing the crude plasma membranes was suspended to a concentration of 2–4 mg/ml in a solution of 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, and 10 mM Tris-HCl, pH 7.6. A volume of 50 μl of this suspension was then ADP-ribosylated (13–19) for 30–45 min at 31°C with the addition of 10 μl of (α-^{32}P)NAD (10 μCi), 20 μl of "activated" cholera toxin (4 μg) and 20 μl of a buffer containing 10 mM ATP, 5 mM nicotinamide, 5 μM NAD, 5 μg of pyruvate kinase, 75 mM phosphoenol pyruvate, and 250 mM potassium phosphate, pH 7. Choleragen was "activated" by incubating 0.1 mg of the toxin in 500 μl of a solution containing 2 mg ovalbumin, 135 mM NaCl, 20 mM DTT, and 40 mM HEPES, pH 7.5, for 10 min at 31°C (12). After ADP-ribosylation, membranes were pelleted in a Beckman microfuge (10,000 *g* for 5 min). The pellets were washed twice with the solution used to suspend the original pellet. The final pellet (100–200 μg) was then solubilized in 50 μl of 2% SDS, 5% 2-mercaptoethanol, 2% Nonidet P-40, and 10% glycerol for 30 min at 50°C before electrophoresis (9) on a 10% SDS gel with a 3% stacking gel. Methods for gel fixation, staining, and destaining were described by Fairbanks et al. (20). Gels were then sliced with a Bio-Rad gel slicer (Bio-Rad Laboratories, Richmond, CA) and the presence of ^{32}P determined with a liquid scintillation counter.

Determination of cAMP and Adenylate Cyclase Activity

After removal of the medium, cAMP was extracted from the cells with 2.0 ml of 0.1 M HCl for 20 min at 37°C. After lypholization, the residue was dissolved in 3 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 8 mM theophylline and 6 mM 2-mercaptoethanol and assayed for cAMP by a slight modification (21) of the cAMP-binding protein method described by Brown et al. (22).

The response of adenylate cyclase to various effectors was determined in membranes that were prepared as described above for ADP-ribosylation. Portions of the membrane suspension containing 65 μg of protein were assayed for adenylate cyclase activity in a final volume of 100 μl according to the procedure of Salomon (23), except that chloride rather than acetate salts of Tris and Mg^{2+} were used, the concentrations of Mg^{2+} and ATP were doubled, and GTP was omitted except for reactions in which choleragen was the effector. In the latter instance, choleragen together with NAD and GTP was used to activate MLTC-1 membranes as previously described (12).

Other Methods

Chromosome number was determined by the method of Moorhead et al. (24). The determination of progesterone was done with a commercially available radioimmunoassay kit supplied by New England Nuclear (Boston, MA). Samples were assayed without performing the extraction or gel filtration steps described in the protocol as they were found to be unnecessary. Quantification of protein was done by the procedure of Lowry et al. (25) unless interfering substances were present. In such cases, protein was assayed with Coomassie Brilliant Blue as described by Bradford (26).

Materials

The hCG (11,600 IU/mg, CR 119) was provided by R. Canfield (Columbia University, through the Center for Population Research of the National Institute of Child Health and Human Development). The hCG was iodinated by Meloy Industries (Bethesda, MD) as described by Ketelslegers and Catt (27) to a specific

activity of 38–49 μCi/μg. Choleragen was obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY). [α-^{32}P]NAD (200–400 Ci/mmol) and (α-^{32}P)ATP (20–25 Ci/mmol) were obtained from ICN Nutritional Biochemicals (Cleveland, OH). [2,8-^{3}H]cAMP and [methyl-^{14}C]methylated ovalbumin and globulins were from New England Nuclear. Bovine LH (NIH-LH-B10, M_r = 28,260, 1.06 U/mg relative to the reference standard NIH-LH-S1), bovine thyrotropin (NIH-TSH-B9, M_r = 28,000, 21.1 IU/mg) and bovine follitropin (NIH-FSH-B1, M_r = 33,000, 0.49 IU/mg) were obtained through the Pituitary Hormone Distribution Program (National Institute of Arthritis, Metabolism, and Digestive Diseases). Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Co. (Rockford, IL). All other materials were reagent grade.

RESULTS

Adaptation of Leydig Tumor Cells to Culture with Retention of Hormone Responsiveness

The Leydig tumors that had been stored in liquid nitrogen were slow to grow in vivo and required ~6 wk before the tumor became noticeable as a lump on the side of the animals. However, after several serial passes of the tumor, transplantation could be done at intervals of 2 wk. The survival rate for recipients of the tumor was almost 100%, and of the surviving mice ~80% developed tumors.

Tumor cells transferred from animals to culture dishes grew rapidly and eventually gave rise to nine clonal cell lines. These cell lines were screened for their ability to bind hCG and to generate cAMP in response to the hormone. The uncloned cells as well as cells of the clonal lines exhibited hCG binding and responsiveness (Table I). The cell line that is designated as MLTC-1 was used for experiments reported here as it was the first line available in sufficient quantities.

Cell Biology of the MLTC-1 Line

MLTC-1 as they appear under normal growth conditions are shown in Fig. 1. When plated at a density of 10^4 cells/cm^2, they begin to grow exponentially after a lag period of 1 to 2 d. A doubling of cell number occurred every 35–40 h and the cells continued to grow rapidly until reaching a density of ~2×10^5 cells/cm^2 at which point the rate of growth was slowed. It has since been found that the lag phase can be virtually eliminated by increasing the serum content of the medium; however, in the interest of consistency all experiments have been done with cells grown in the medium used to originally establish the clone. Chromosome spreading showed that these cells were polyploid having 95 ± 4 chromosomes per cell.

TABLE I

Binding of hGC to and cAMP Generation by Cloned and Uncloned Leydig Tumor Cells

Cells	hCG Bound*	cAMP Generation‡	
		Basal	+hCG
	fmol/10 cells		*nmol/10 cells*
Uncloned	22.0 ± 1.0	0.05 ± 0.02	2.2 ± 0.1
MLTC-1	28.3 ± 1.1	0.09 ± 0.03	2.7 ± 0.3
MLTC-2	39.1 ± 0.1	0.06 ± 0.00	4.24 ± 0.03
MLTC-3	28.9 ± 0.3	0.19 ± 0.00	3.13 ± 0.06
MLTC-4	56.0 ± 1.9	0.20 ± 0.07	4.7 ± 0.7
MLTC-5	56.6 ± 0.7	0.13 ± 0.00	3.4 ± 0.8
MLTC-6	74.5 ± 2.1	0.3 ± 0.1	6.1 ± 1.3
MLTC-7	48.8 ± 0.1	0.13 ± 0.04	5.0 ± 0.9
MLTC-8	16.8 ± 0.6	0.38 ± 0.00	2.0 ± 0.3
MLTC-9	14.0 ± 0.5	0.22 ± 0.00	1.6 ± 0.2

Uncloned and cloned (MLTC-1–MLTC-9) cells derived from the M56480P murine Leydig tumor grown in 25-cm^2 flasks (2×10^6 cells/flask) were assayed for ^{125}I-hCG binding and cAMP production as described under Materials and Methods. Values are the mean and range of duplicates for a single experiment.

* Data represent hCG bound by cells during a 1-h incubation in the presence of 5×10^{-10} M ^{125}I-hCG. Nonspecific binding was <10% and has been subtracted.

‡ Data represent cAMP generated during a 1-h incubation in the presence or absence of 5×10^{-10} M hCG.

Characteristics of hCG Binding to MLTC-1 Cells

The binding of ^{125}I-hCG to MLTC-1 cells was highly specific. Table II demonstrates that of the several peptide hormones tested only hCG and LH inhibited ^{125}I-hCG binding. The effect of LH was expected as it binds to the same receptors on normal Leydig cells as hCG (10). The small amount of inhibition by thyroid-stimulating hormone (TSH) was attributed to contamination of this preparation with LH. Binding of hCG to intact cells appeared to be of high affinity. Scatchard analysis indicated a K_d of 5.4 ± 0.6 × 10^{-10} M (n = 4). Binding kinetics with intact cells should be considered with caution, as equilibrium may not be reached due to receptor internalization and recycling (28) and receptor number may be altered further because hCG is known to induce down regulation of its receptors (29).

Binding of hCG to MLTC-1 Membrane Preparations

Initial experiments indicated that binding of very low concentrations of hCG (20 pM) to crude membranes reached

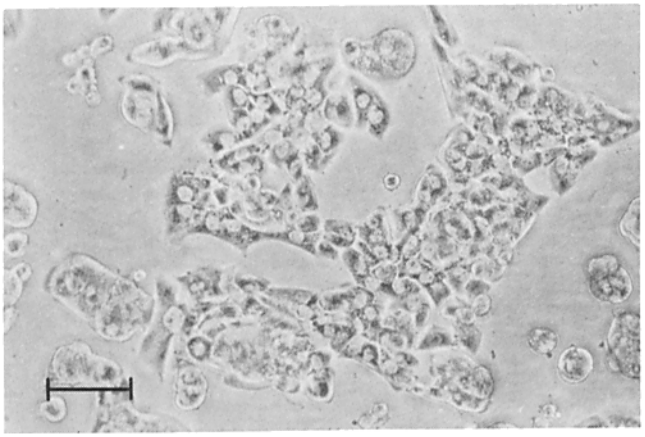

FIGURE 1 Phase-contrast micrograph of MLTC-1 cells. MLTC-1 at a density of 2×10^4 cells/cm^2 were photographed 4 d after subculturing. Bar, 100 μm. × 115.

TABLE II

Hormone Binding Specificity of MLTC-1

Hormone	% Inhibition of binding
hCG	100 ± 1
LH	58 ± 8
Thyrotropin	6.7 ± 0.6
Follitropin	1 ± 4
Insulin	1.3 ± 0.3

MLTC-1 cells in 25-cm^2 flasks were incubated for 1 h with 5×10^{-10} M ^{125}I-hCG in the absence and presence of 10^{-8} M of the indicated hormone (concentration assumes 100% purity of the hormone) and assayed for bound ^{125}I-hCG as described under Materials and Methods. Each value represents the mean and range of duplicates in a single experiment. 100% equals displaceable counts (21,000 − 1,400 = 19,600) out of 21,000 initially bound in the presence of tracer alone.

equilibrium by 2 h at 37°C. There was no evidence of proteolysis, as binding curves were the same in the presence or absence of phenylmethyl sulfonylfluoride, a potent protease inhibitor. A K_d of 1.0 ± 0.2 × 10^{-10} M (n = 5) (Fig. 2) was determined for hCG binding to disrupted cells and was independent of the type of preparation (whole homogenate or crude membrane pellet), the length of incubation (2 or 14 h), or the means of membrane collection after hormone binding (centrifugation or filtration). In determining receptor number, crude homogenates were incubated with increasing concentrations of hCG for 2 h at 37°C and collected by filtration, a technique found to be as effective as centrifugation at 100,000 g for 1 h, suggesting that recovery of membranes was essentially complete. A value 100 fmol/mg protein was obtained. Based on a ratio of 2.1 × 10^6 cells/mg crude homogenate protein, it was estimated that there were 29,000 ± 3,000 binding sites/cell. As a cautionary note, this should be taken as the lowest estimate since homogenization may have masked or destroyed some receptors.

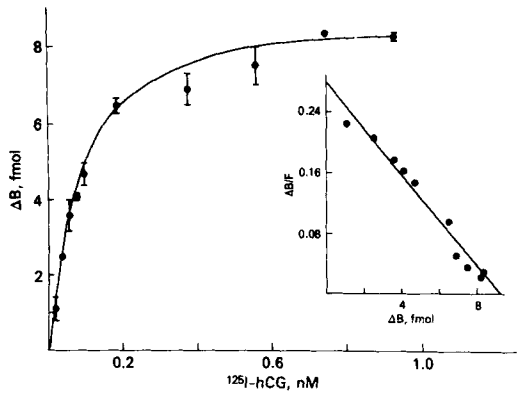

FIGURE 2 Binding of hCG to membranes isolated from MLTC-1. Membranes from MLTC-1 cells and assayed for ^{125}I-hCG binding with increasing concentrations of hCG as described under Materials and Methods. Data represent duplicate determinations for specific binding in a typical experiment with error bars showing the range.

Physical Parameters of the hCG Receptor from MLTC-1 Cells

Bound ^{125}I-hCG was covalently cross-linked to its receptor on MLTC-1 membranes with DSS (8). After extracting the complex with detergent, it was analyzed by sucrose density gradient centrifugation (Fig. 3*A*); a sedimentation coefficient of 8.5 S was obtained. This value was identical to that of the receptors from normal murine Leydig cells (Fig. 3*B*).

SDS PAGE was also used to determine the respective molecular weights of the cross-linked hormone-receptor complexes from normal and tumor cells (Fig. 4). Based on similar experiments (8), it was concluded that peak I in Fig. 4 represents the receptor cross-linked to the intact, cross-linked hCG hormone. In both normal Leydig cells and MLTC-1, the molecular weight of the receptor as determined by subtracting the molecular weight of the intact hormone (peak III) from the molecular weight of the hormone-receptor complex was 95,000. Peak II represents the α-subunit of hCG cross-linked to the hCG receptor. All of the free α-subunit and most of the intact, cross-linked hormone has been eliminated by gel filtration on Sephadex G-200 before electrophoresis.

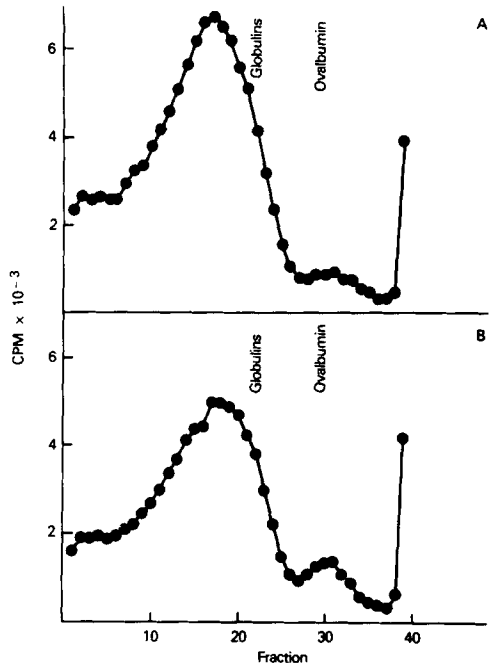

FIGURE 3 Sedimentation behavior of cross-linked hCG-receptor complex from MLTC-1 and normal murine Leydig cells. Membranes were incubated with ^{125}I-hCG, treated with the cross-linker DSS, and extracted with 1% Triton X-100 as described under Materials and Methods. The extracted cross-linked hCG-receptor complexes from normal murine Leydig cells (*A*) and MLTC-1 (*B*) were sedimented on 5-20% linear sucrose gradients centrifuged at 120,000 g for 16 h. Ovalbumin and globulins were used as markers.

Stimulation of Adenylate Cyclase in MLTC-1 Cells and Membranes

The production of cAMP by MLTC-1 in response to hCG was time- and concentration-dependent. In the presence of 5 × 10^{-10} M hCG, the amount of cAMP within the cells increased linearly with time up to 30 min and then declined slowly (Fig. 5*A*). Concentrations of hCG as low as 10^{-11} M stimulated cAMP production with a maximal rate occurring at hCG concentrations approximately an order of magnitude higher (Fig. 5*B*). Half-maximal stimulation occurred at 5 × 10^{-11} M (Fig. 5*B*) and at 7 × 10^{-11} M when cells were incubated for only 30 min. Membranes prepared from MLTC-1 cells contained an adenylate cyclase that was stimulated by hCG and LH as well as guanine nucleotides, NaF and "activated choleragen" (Table III). Activation of adenylate cyclase by choleragen also was obtained by incubating the intact cells with the toxin (data not shown).

A substantial increase in the radiolabeling of certain MLTC-1 membrane proteins occurred in the presence of choleragen when [α-^{32}P]NAD was used as a substrate for the toxin (Fig. 6). It was presumed, based on the work of other investigators (13–19), that the toxin had catalyzed the ADP-ribosylation of these proteins. The principal recipient of the ADP-ribosyl moiety had a molecular weight of 53,900 ± 800 as determined on the basis of three separate experiments.

Hormone-stimulated Steroid Production in MLTC-1

As in the case for the tumor grown in vivo (6), MLTC-1 is capable of producing the steroid progesterone in response to hCG. The time course of progesterone production in these cells is shown in Fig. 7. When the cells were exposed to hCG, the

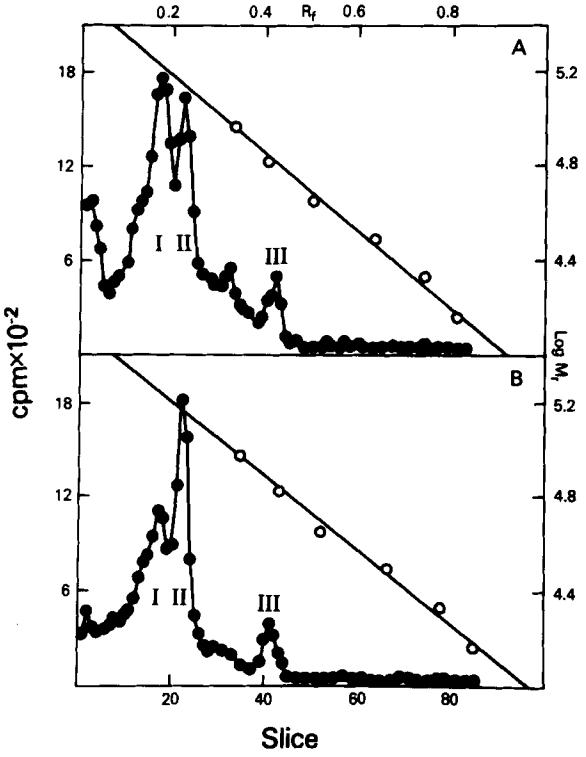

FIGURE 4 Molecular weight determination by SDS PAGE of cross-linked hCG-receptor from MLTC-1 and normal murine Leydig cells. Membranes were incubated with ^{125}I-hCG, treated with the cross-linker DSS, and dissolved in SDS as described under Materials and Methods. After Sephadex G-200 column chromatography, the cross-linked complexes from normal murine Leydig cells (*A*) and MLTC-1 (*B*) were analyzed by SDS PAGE. The mobilities of proteins of known molecular weight (lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, and phosphorylase B) are indicated (empty circles). The gel was sliced into 1-mm lengths and assayed for ^{125}I in a gamma counter. Peak I represents the cross-linked complex composed of intact hormone and receptor, peak II represents the α-subunit of hCG cross-linked to the receptor, and peak III the intact, cross-linked hormone.

production of progesterone increased linearly with time for 1.5 h and most of the steroid was released into the medium. Testosterone generation was not measured since it is reportedly not induced by hormone (6).

DISCUSSION

Normal Leydig cells located *in situ* comprise a very low percentage of the total cell mass of the testis, and obtaining large quantities of highly enriched and viable cells is difficult. Experimental manipulation of the cells in vivo is also difficult as the success of an experiment may depend upon injected effectors reaching their target unaltered and in sufficient quantities to be effective, and isolation of Leydig cells from the intact organ remains a problem. The development of transplantable tumors made it possible to isolate large numbers of Leydig cells (6, 30), but their viability is also limited. In addition, the metabolic state of these tumor cells will be influenced by the host and the age of the tumor. Recent establishment of the hCG/LH-responsive MLTC lines (3) and other cell lines isolated from the same tumor by Ascoli (4, 5) circumvents these problems. Although all of the cell lines established in this laboratory have receptors for hCG and produce cAMP in response to the hormone, only the properties of the MLTC-1 cell line are reported.

The principal disadvantage of this system arises from the fact that the tumor cells are different from normal Leydig cells in being polyploid. The cells, however, seem to have retained the components of the hormone-responsive adenylate cyclase system and are capable of translating the hormonal message into the production of steroids as do normal cells. Of the hormones tested, only LH and hCG could block the binding of ^{125}I-hCG to MLTC-1, suggesting the presence of a receptor with specificity for either of these hormones. The experiments presented here, however, do not rule out the existence of

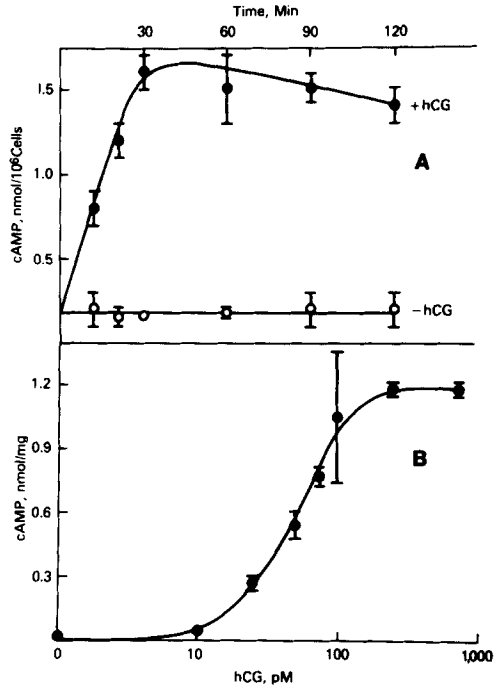

FIGURE 5 Stimulation of cAMP production in MLTC-1 by hCG. Cells were incubated in complete growth medium containing 0.25 mM 3-isobutyl-1-methyl-xanthene at 37°C for indicated times in the absence (empty circles) and presence (filled circles) of 5×10^{-10} M hCG (*A*). Measurement of the dose response of MLTC-1 to hCG was done in the same manner except that the time of incubation was fixed at 1 h and the concentration of hCG was varied as indicated (*B*). The cells then were analyzed for intracellular cAMP as described under Materials and Methods. Values represent tbe mean ± SD for quadruplicate determinations in a single experiment.

TABLE III

Adenylate Cyclase Activity in MLTC-1 Membranes

Effector	Activity*
None	100 ± 10
hCG	620 ± 50
LH	590 ± 50
NaF	3,260 ± 80
GPP(NH)P	1,360 ± 50
Choleragen	1,160 ± 70

Membranes were prepared from MLTC-1 cells and assayed for adenylate cyclase activity as described under Materials and Methods. Each assay contained 65 μg of protein and was incubated for 10 min at 31°C. Effector concentrations were 10 nM hCG, 10 nM LH, 10 mM NaF, and 50 μM GPP(NH)P. In addition, membranes were activated with 0.3 μM choleragen, 2 mM NAD and 100 μM GTP (11) and assayed for cyclase activity. Activity in membranes treated the same way in the absence of choleragen was 100 ± 6 pmol/10 min/mg protein.

* Adenylate cyclase activity for MLTC-1 membranes is reported as pmol/10 min/mg protein and represents the mean ± SD for triplicates in a single experiment.

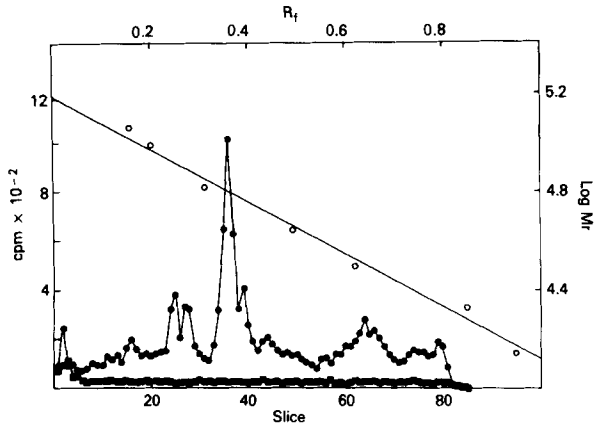

FIGURE 6 Choleragen-mediated ADP-ribosylation of membranes from MLTC-1. Membranes were prepared from MLTC-1 and incubated with (α-32P)NAD in the presence (filled circle) and absence (filled square) of "activated" choleragen as described under Materials and Methods. The membranes were dissolved in SDS and analyzed by SDS PAGE. Incorporation of ^{32}P was determined by counting gel slices in a liquid scintillation counter and molecular weights were determined relative to the standard proteins (empty circles) as indicated in the legend to Fig. 5.

receptors for hormones other than LH or hCG.[1] Scatchard analysis of data based on isolated membranes indicated the presence of a single class of hCG receptors with a K_d of 1.0 ± 0.2 × 10^{-10} M which compares favorably with values reported for the tumor grown in vivo (6) and for membranes isolated from normal rat Leydig cells (10, 31). Barring significant loss of receptor binding activity during homogenization, it was estimated that intact MLTC-1 bind the equivalent of 29,000 ± 3,000 hCG molecules/cell. This number is substantially greater than values for either normal Leydig cells isolated from testes by collagenase dispersion (32) or Leydig tumor cells grown in vivo (6). Such a large difference may be the result of using different preparations. The use of a homogenate in this case hopefully avoids the difficulties of receptor turn-over that are likely to occur when using intact cells (6, 32) for estimation of receptor number.

When Scatchard analysis was used to determine the K_d of hCG binding to intact cells, a value of 5.4 ± 0.6 × 10^{-10} M was obtained. This value was similar to that observed by Ascoli (4) for his clonal Leydig cell lines but significantly higher than values for isolated membranes. Similar discrepancies have been reported for β-adrenergic receptors (33, 34). Data for K_d in intact cells are regarded with caution since a true equilibration between hormone and receptor is unlikely to be established due to the dynamic properties of the living cell. However, the number is useful in showing that, at least qualitatively, the intact cell has an apparent high affinity for hormone.

In terms of physical parameters, the hCG receptor from MLTC-1 appears to be identical to that from normal murine Leydig cells. Analysis on SDS PAGE showed that both receptors have an apparent molecular weight (M_r) of 95,000. Although this is the predominant species, the high molecular weight region of the polyacrylamide gels presented in Fig. 4 suggests that there may be some heterogeneity in the receptor as has been shown by Ji and co-workers (35–37) for ovarian granulosa cells. On sucrose density gradients, cross-linked hCG receptors solubilized from normal Leydig and MLTC-1 cells displayed a sedimentation coefficient of 8.5 S. As previously suggested for the hCG receptor from rat testes (8), the detergent-solubilized murine receptor may exist as a complex which can be dissociated by SDS.

These specific receptors are functional as intact cells accumulated cAMP in response to hCG. The rate of cAMP accumulation was time-dependent, reaching a maximum by 30 min and then declining. This latter effect appears to be a consequence of hormone-mediated desensitization (unpublished observations). Hormone-stimulated cAMP accumulation was dose dependent with an apparent K_a of 5 × 10^{11} M. Because a true equilibrium may not have been obtained and other processes such as desensitization and downregulation are occurring, a direct comparison between the K_d for hCG binding to intact cells and the K_a for hCG-stimulated cAMP production may not be appropriate. Both processes, however, appear to be of high-affinity.

Stimulation of adenylate cyclase also was observed in isolated membranes. The enzyme activity increased in the presence of hCG and LH as well as NaF, guanine nucleotides, and choleragen. With MLTC-1 membranes, a sixfold stimulation of cyclase activity was obtained with LH and hCG in the absence of added guanine nucleotides. This is in contrast to the findings of Dufau et al. (38), who observed that adenylate cyclase activity in rat testicular membranes was insensitive to LH unless guanine nucleotides were added to the assay. Thus it appears that MLTC-1 membranes contain endogenous guanine nucleotides.

It is generally accepted that hormone-sensitive adenylate cyclases are composed of at least three components: a hormone receptor, a catalytic subunit, and a regulatory component that binds guanine nucleotides (2, 39). It has also been shown that choleragen activated adenylate cyclase by ADP-ribosylation of the regulatory component (13–15). Thus, stimulation of adenylate cyclase by guanine nucleotides and choleragen is consistent with the presence of the regulatory component in MLTC-1 cells. Its presence was directly demonstrated by the ADP-ribosylation of a 54,000 M_r protein by incubating MLTC-1 membranes with choleragen and (α-^{32}P)NAD. In some cells, a 42,000 M_r protein is ADP-ribosylated by the toxin (13, 14,

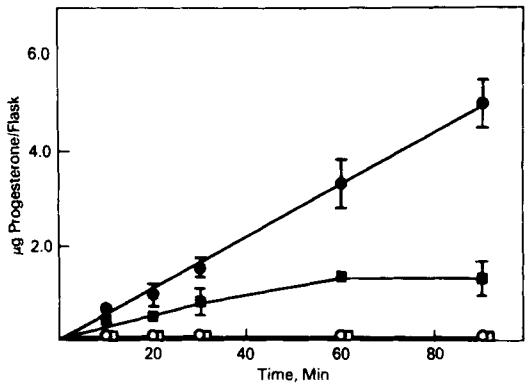

FIGURE 7 Stimulation of progesterone production in MLTC-1 cells by hCG. Cells were incubated in serum-free medium at 37°C in an atmosphere of 5% CO_2 in air in the absence (empty circle, empty square) or presence (filled circle, filled square) of 5 × 10^{-10} M hCG. Progesterone contained in the cells (empty square, filled square) or released into the medium (empty circle, filled circle) was determined as described under Materials and Methods. Each flask contained 1.3 ± 0.1 mg of cell protein. Values represent the mean and range for a single experiment.

[1] MLTC-1 cells accumulated cAMP in response to prostaglandin E1 but not isoproterenol (unpublished observations).

19); in other cells, this protein and a protein of higher molecular weight (52,000–55,000 M_r) are labeled (15–17); and, in a few cells, only the higher molecular weight form is present (17, 18).

Hormone stimulation of normal cells with hCG eventually leads to the production of steroid hormones, principally testosterone (32). The M548OP Leydig tumor, however, produces progesterone in response to hCG (6). Testosterone is synthesised in small quantities and its synthesis is not induced by hormone (6). As expected, the MLTC-1 cell line derived from this tumor also produced progesterone in response to hCG. Most of the steroid was released into the medium. In the absence of hCG, no detectable progesterone was generated. Progesteone also was the end product of hormone stimulation in the cell lines developed from the same tumor by Ascoli (4, 5).

Experiments reported herein have been done with cells passed <30 times since cloning; and, although no changes have been noted, they may occur with continual passage of the cells. It is evident, however, that MLTC-1 cultures of low passage have retained the components of the adenylate cyclase system. It is also evident that the MLTC-1 line is capable of responding to hCG by producing steroids although the principal end product is progesterone rather than testosterone. Other murine Leydig tumor cell lines have been established, but they do not respond to gonadotropins (40) and lack hCG receptors.[2] These properties are unique to cell lines derived from the M548OP Leydig tumor in both this laboratory and that of Ascoli (4, 5) and offer an opportunity to study various aspects of this hormone-responsive system while using the advantages of cell culture.

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REFERENCES

1. Pierce, J. G., and T. F. Parsons. 1981. Glycoprotein hormones: structure and function. *Ann. Rev. Biochem.* 50:465–495.
2. Ross, E. M., and A. G. Gilman. 1980. Biochemical properties of hormone-sensitive adenylate cyclase. *Ann. Rev. Biochem.* 49:533–564.
3. Rebois, R. V. 1981. Establishment and properties of a gonadotropin-responsive clonal murine Leydig tumor cell line. *Fed. Proc.* 40:1879(Abstr.).
4. Ascoli, M. 1981. Regulation of gonadotropin receptors and gonadotropin responses in a clonal strain of Leydig tumor cells by epidermal growth factor. *J. Biol. Chem.* 256:179–183.
5. Ascoli, M. 1981. Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology.* 108:88–95.
6. Ascoli, M., and D. Puett. 1978. Gonadotropin binding and stimulation of steroidogenesis in Leydig tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:99–102.
7. Dufau, M. L., E. H. Charreau, and K. J. Catt. 1973. Characteristics of a soluble gonadotropin receptor from the rat testes. *J. Biol. Chem.* 248:6973–6982.
8. Rebois, R. V., F. Omedeo-Sale, R. O. Brady, and P. H. Fishman. 1981. Crosslinking of human chorionic gonadotropin to its receptor in rat testes. *Proc. Natl. Acad. Sci. U. S. A.* 78:2086–2089.
9. Laemmli, U. K. 1971. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
10. Catt, K. J., J. M. Ketelslegers, and M. L. Dufau. 1976. Receptors for gonadotropic hormones. *In* Methods in Receptor Research. M. Blecher, editor. Marcel Dekker, New York. 175–250.
11. Pacuszka, T., J. C. Osborne, Jr., R. O. Brady, and P. H. Fishman. Interaction of human chorionic gonadotropin with membrane components of rat testes. *Proc. Natl. Acad. Sci. U. S. A.* 75:764–768.
12. Fishman, P. H., P. Mallorga, and J. F. Tallman. 1981. Catecholamine-induced desensitization of adenylate cyclase in rat glioma C6 cells: evidence for a specific uncoupling of beta-adrenergic receptors from a functional regulatory component of adenylate cyclase. *Mol. Pharmacol.* 20:310–318.
13. Cassel, D., and T. Pfeuffer. 1978. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* 75:2669–2673.
14. Gill, D. M., and R. Meren. 1978. ADP-Ribosylation of membrane proteins catalyzed by cholera toxin: basis of the action of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* 75:3050–3054.
15. Johnson, G. L., H. R. Kaslow, and H. R. Bourne. 1978. Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. *J. Biol. Chem.* 253:7120–7123.
16. Northrup, J. K., P. C. Sternweiss, M. D. Smigel, L. S. Schleifer, E. M. Ross, and A. G. Gilman. 1980. Purification of the regulatory component of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* 77:6516–6520.
17. Kaslow, H. R., D. Cox, V. E. Groppi, and H. R. Bourne. 1981. An M_r = 51,000 peptide can mediate effects of cholera toxin on adenylate cyclase in intact cells. *Mol. Pharmacol.* 19:406–410.
18. Beckner, S. K., and M. Blecher. 1981. Endogenous and cholera toxin-catalyzed ADP-ribosylation of a plasma membrane protein of RL-PR-C cloned rat hepatocytes. *Biochim. Biophys. Acta.* 673:477–486.
19. Downs, W. R., Jr., S. A. Reen, M. A. Levine, G. D. Aurbach, and A. M. Spiegel. 1981. Cholera-toxin-dependent ADP-ribosylation of the adenylate cyclase regulatory protein in turkey erythrocyte membranes. *Arch. Biochem. Biophys.* 209:284–290.
20. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2616.
21. Smith, C. C., J. F. Tallman, R. M. Post, D. P. van Kammer, D. C. Jimerson, G. L. Brown, B. R. Brooks, and W. E. Bunney, Jr. 1976. An examination of base line and drug-induced levels of cyclic nucleotides in the cerebrospinal fluid of control and psychiatric patients. *Life Sci.* 19:133–136.
22. Brown, B. L., J. D. M. Albono, R. P. Ekins, A. M. Sgherzi, and W. Tampion. 1971. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* 121:561–562.
23. Salomon, Y. 1979. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* 10:35–55.
24. Moorhead, P. S., P. C. Nowell, W. J. Mellman, D. M. Battips, and D. A. Hungerford. 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell. Res.* 20:613–616.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–27.
26. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
27. Ketelslegers, J. M., and K. J. Catt. 1974. Receptor binding properties of ^{125}I-hFSH prepared by enzymatic iodination. *J. Clin. Endocrinol. Metab.* 39:1159–1162.
28. Wiley, H. S., and D. D. Cunningham. 1981. A steady state model for analyzing the cellular binding, internalization and degradation of polypeptide ligands. *Cell.* 25:433–440.
29. Catt, K. J., J. P. Harwood, G. Aguilera, and M. L. Dufau. 1979. Hormonal regulation of peptide receptors and target cell responses. *Nature (Lond.).* 280:109–116.
30. Cooke, B. A., L. M. Lindh, F. H. A. Janszen, M. J. A. van Driel, C. P. Bakker, M. P. I. van der Plank, and H. J. van der Molen. A Leydig cell tumor: a model for the study of lutropin action. *Biochim. Biophys. Acta.* 583:320–331.
31. Catt, K. J., T. Tsuruhara, C. Mendelson, J. M. Ketelslegers, and M. L. Dufau. 1970. *In* Hormone Binding and Target Cell Activation in the Testis. A. R. Means and M. L. Dufau, editors. Plenum Publishing Corp., New York. 145–165.
32. Mendelson, C., M. L. Dafau, and K. J. Catt. 1975. Gonadotropin binding and stimulation of cyclic adenosine 3′:5′-monophosphate and testosterone production in isolated Leydig cells. *J. Biol. Chem.* 250:8818–8823.
33. Terasaki, W. L., and G. Brooker. 1978. [^{125}I]Iodohydroxybenzylpindolol binding sites on intact rat glioma cells. *J. Biol. Chem.* 253:5418–5425.
34. Pittman, R. N., and P. B. Molinoff. 1980. Interactions of agonists and antagonists with β-adrenergic receptors on intact L6 muscle cells. *J. Cyclic Nucleotide Res.* 6:421–435.
35. Ji, I., and T. H. Ji. 1980. Macromolecular photoaffinity labeling of the lutropin receptor on granulosa cells. *Proc. Natl. Acad. Sci. U. S. A.* 77: 7167–7170.
36. Ji, I., and T. H. Ji. 1981. Both α and β subunits of human choriogonadotropin photoaffinity label the hormone receptor. *Proc. Natl. Acad. Sci. U. S. A.* 78:5465–5469.
37. Ji, I., B. Y. Yoo, C. Kaltenbach, and T. H. Ji. 1981. Structure of the lutropin receptor on granulosa cells. *J. Biol. Chem.* 256:10853–10858.
38. Dufau, M. L., A. J. Baukal, and K. J. Catt. 1980. Hormone-induced guanyl nucleotide binding and activation cyclase in the Leydig cell. *Proc. Natl. Acad. Sci. U. S. A.* 77:5837–5841.
39. Rodbell, M. 1980. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.).* 284:17–22.
40. Wolff, J., and G. H. Cooke. 1975. Choleragen stimulates steroidogenesis and adenylate cyclase in cells lacking functional hormone receptors. *Biochim. Biophys. Acta.* 413:283–290.

[2] The murine Leydig tumor cell line, I-10, did not bind ^{125}I-hCG (unpublished observations).