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
caracterized with regard to the gonadotropin-responsive adenylate cyclase system. The
binding of 125I-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 ± 0.2 x 10^-10 M. The receptors
appeared identical to those from normal murine Leydig cells when analyzed by SDS PAGE and
succrose 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'-ylmido-diphosphate. Additionally, cholera was found to ADP-
rubosylate a membrane protein of 54,000 daltons. This protein resembles the proposed guanine
nucleotide regulatory component in both size and cholera-dependent reactivity. These data
suggest that MLTC-1 cells possess a gonadotropin-responsive adenylate cyclase system con-
sisting 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 dem-
strate 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 hor-
mone 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 succrose
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, Fla. After several serial transplants in C3HT/Bi/
63 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 semistereile conditions into anesthetized mice was achieved by subcutaneous injection of the tissue just posterior to the rib cage with a 27-gauge 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-cm² 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 by detaching with forceful pipetting. Henceforth, cells were grown in 25-cm² 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% CO₂ in air at 37°C. Subculturing was achieved by replacement of the medium with a minimal amount of 25% fetal calf serum in Ca²⁺/Mg²⁺-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 subcultured into new culture flasks. For the purpose of cloning, cells were diluted to an average density of two cells/ml, and 0.1-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-I 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 120,000 × 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 x 10⁵ disintegrations/min (dpm) of ¹²⁵I-hCG (5 x 10⁶ cpm) 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 disuccinimidyldiethyl carbonate (DSC) as described by Rebois et al. (8) and was analyzed either by SDS PAGE (9, 10) 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 (³⁵S-labeled ovalbumin and globulins), the extracts were placed on 5-m1 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 ¹²⁵I and ¹³¹I 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 µg of membrane protein was centrifuged for 30 min at 100,000 g in a Beckman airfuge (Beckman Instruments, Inc., Spincos Div., Palo Alto, CA). The resulting supernatant was passed over a 0.9 x 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**

Hormone binding to a clonal Leydig tumor cells was performed in culture flasks. The growth medium was replaced with 2 ml of fresh medium containing ²⁴¹I-hCG. Unlabeled hCG or other hormones were present in some experiments. After incubating for 1 h at 37°C, the culture was washed twice, each time with 2 ml of Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg²⁺ (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.

The dissociation constant (Kd) for hCG was determined from 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 ²⁴¹I-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). The pellets were washed twice, each time with 2 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.

**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 0.2 M sucrose, 5 mM MgCl₂, 1 mM DTT, and 10 mM Tris-HCl, pH 7.6. A volume of 50 µl of this suspension was then incubated (13-19) for 30-45 min at 31°C with the addition of 10 µl of [³²P]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 mM 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). Cells were then sliced with a Bio-Rad gel slicer (Bio-Rad Laboratories, Richmond, CA) and the presence of [³²P] 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 lyophilization, the residue was dissolved in 3 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 8 mM theophylline and 6 mM 2-mercaptopropanol 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 0.1 ml containing 65 mM of a buffer of Salomon (23), except that chloride rather than acetate salts of Tris and Mg²⁺ were used, the concentrations of Mg²⁺ 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 at the National Institute of Child Health and Human Development). The hCG was iodinated by Mely Industries (Bethesda, MD) as described by Ketelslegers and Catt (27) to a specific...
activity of 38–49 µCi/µg. Cholera toxin was obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY). [α-32P]NAD (200–400 Ci/mmol) and [α-32P]ATP (20–25 Ci/mmol) were obtained from ICN Nutritional Biochemicals (Cleveland, OH). [3H]-cAMP and [methyl-3H]methylated ovalbumin and globulins were from New England Nuclear. Bovine LH (NIH-LH-B10, M = 28,260, 1.06 U/mg relative to the reference standard NIH-LH-S1), bovine thyrotropin (NIH-TSH-B9, M = 28,000, 21.1 IU/mg) and bovine follitropin (NIH-FSH-B1, M = 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 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/cm², 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 × 10^6 cells/cm² 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.

Characteristics of hCG Binding to MLTC-1 Cells

The binding of 125I-hCG to MLTC-1 cells was highly specific. Table II demonstrates that of the several peptide hormones tested only hCG and LH inhibited 125I-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 Kd 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 × 10^6 cells/cm² were photographed 4 d after subculturing. Bar, 100 µm. ×15.](http://example.com/image1.png)

### TABLE I

| Cells               | hCG Bound*   | Basal +hCG (nmol/10 cells) | +hCG (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               | 4.2 ± 0.03           |
| MLTC-3              | 28.9 ± 0.3   | 0.19 ± 0.00               | 3.13 ± 0.06          |
| MLTC-4              | 55.0 ± 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-cm² flasks (2 × 10^6 cells/flask) were assayed for 125I-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 125I-hCG. Nonspecific binding was <10% and has been subtracted.

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

### TABLE II

| 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-cm² flasks were incubated for 1 h with 5 × 10^-10 M 125I-hCG in the absence and presence of 10^-6 M of the indicated hormone (concentration assumes 100% purity of the hormone) and assayed for bound 125I-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 $1.0 \pm 0.2 \times 10^{-10}$ 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 $100 \text{ fmol/mg protein}$ was obtained. Based on a ratio of $2.1 \times 10^6$ cells/mg crude homogenate protein, it was estimated that there were $29,000 \pm 3,000$ binding sites/cell. As a cautionary note, this should be taken as the lowest estimate since homogenization may have masked or destroyed some receptors.

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. 3A); a sedimentation coefficient of 8.5 S was obtained. This value was identical to that of the receptors from normal murine Leydig cells (Fig. 3B).

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 a-subunit of hCG cross-linked to the hCG receptor. All of the free a-subunit and most of the intact, cross-linked hormone has been eliminated by gel filtration on Sephadex G-200 before electrophoresis.

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 $5 \times 10^{-10}$ M hCG, the amount of cAMP within the cells increased linearly with time up to 30 min and then declined slowly (Fig. 5A). 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. 5B). Half-maximal stimulation occurred at $5 \times 10^{-11}$ M (Fig. 5B) and at $7 \times 10^{-11}$ 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 choleraen" (Table III). Activation of adenylate cyclase by choleraen 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 choleraen when [alpha$^{32}$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 $\pm 800$ 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 crosslinker 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 $\alpha$-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 $5 \times 10^{-10}$ 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 the mean ± SD for quaduplicate determinations in a single experiment.

Table III

| 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 nM 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 quaduplicate determinations in a single experiment.
Counting gel slices in a liquid scintillation counter and molecular from MLTC-1, Membranes were prepared from MLTC-q 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 32P 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.

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 32P 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.

Since 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 Kd 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 Kd for hCG binding to intact cells and the Kd 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, protein by incubating MLTC-1 membranes with choleragen and (α-32P)NAD. In some cells, a 42,000 M, protein is ADP-ribosylated by the toxin (13, 14,

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

I thank Dr. Peter Fishman for helpful discussions and critical review of this manuscript.

The author is a recipient of a National Institutes of Health Postdoctoral Research Fellowship.

Received for publication 19 October 1981, and in revised form 18 March 1982.

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