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Human Chorionic Gonadotropin-Induced Leydig Cell Refractoriness to Gonadotropin Stimulation

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SUMMARY

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A single injection of 500 IU of human chorionic gonadotropin to intact male rats induced steroidogenic refractoriness of Leydig cells to gonadotropin stimulation. Complete refractoriness to human chorionic gonadotropin both *in vivo* and *in vitro* was observed between 12 and 60 hours after the injection. During the same period of time, refractoriness to N^6,O^2-dibutyryl adenosine-3':5'-monophosphate (DbcAMP) *in vitro* was affected to a smaller degree. Human chorionic gonadotropin binding capacity decreased to less than 10% that of the controls within 12 hours, and remained undetectable until 96 hours after the human chorionic gonadotropin injection. Complete responsiveness to DbcAMP was recovered while the response to human chorionic gonadotropin was still very small and the binding sites were still undetectable. Moreover, full responsiveness to human chorionic gonadotropin was obtained when only 25% of the binding sites were measured. Basal, guanylnucleotide, and sodium fluoride stimulated adenylate cyclase activities, as well as cAMP phosphodiesterase activity of Leydig cell particles from human chorionic gonadotropin-treated rats were at all times similar to untreated animals. Ten international units of human chorionic gonadotropin also induced refractoriness, but for a shorter period of time, and the number of human chorionic gonadotropin binding sites was in this case never less than 30% that of controls.

These data suggest that human chorionic gonadotropin-induced refractoriness of Leydig cells steroidogenesis can be related to at least two phenomena: a decrease in the number of binding sites and an abnormality of some step of steroidogenesis beyond cAMP formation.

INTRODUCTION

The hormonal regulation of target tissue responsiveness to specific hormones has been demonstrated for several tissues (1-11). In most cases, the hormones induce a refractory state to further stimulation. Several mechanisms have been postulated to explain such a desensitization: a generation of inhibitors of adenylate cyclase (1), an increased cyclic nucleotide phosphodiesterase activity (6, 7), a modification of the coupling system operating between the binding sites and the adenylate cyclase enzyme (11-13), and a modulation of the number of hormone receptors (2, 3, 5).

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In vivo and *in vitro* desensitization of the ovary by LH/hCG[1] has been reported in several studies (4, 11–15) and has been attributed in part to a decrease of the number of LH/hCG receptors (16). Recently, it has been shown that hCG also induces a decrease of its own receptors in Leydig cells (17–22). The mechanism of this regulation, however, as well as its possible effect on Leydig cell functions remains unknown.

In this study, we present evidence that the refractoriness of Leydig cells following hCG administration is a complex phenomenon which cannot be explained simply by diminution of hormone binding capacity.

MATERIALS AND METHODS

Chemicals and drugs. (1,2-^{3}H)-testosterone (SA: 50 Ci/mmol), (4-^{3}H)-leucine (30 Ci/mmol), [^{3}H]cAMP (27 Ci/mmole) and [^{14}C]adenosine (46.5 mCi/mmol) were obtained from CEA (Saclay, France) and carrier free Na ^{125}I from Amersham, England; hCG came from Organon and purified hCG (15,700 IU/mg) was kindly supplied by Dr. A. Bosch (Oss, Holland); 3-isobutyl-1-methylxanthine came from EGA-Chemie, Brenz, Germany. All other biochemical reagents were obtained from Sigma Chemicals Co., and GMP-P(NH)P from ICN, California.

Animals. Intact male rats aged 46 to 60 days were used in all experiments. For each experimental group two to four rats were used; hCG was dissolved in saline and injected i.m.

In vitro studies with isolated interstitial cells. Interstitial cells were prepared by a modification (19, 23) of the method described by Catt *et al.* (24). The isolated interstitial cells were washed three times with 150 ml of MEM medium (Eurobio) pH 7.4, containing 20 mM HEPES, bovine serum albumin (0.5 mg/ml), and 0.1 mM MIX (3-isobutyl-1-methylxanthine) (buffer A), followed each time by a centrifugation at 100 × *g* for 20 min. The yield of interstitial cells from control and desensitized animals was similar (± 6%).

[1] The abbreviations used are: hCG, human chorionic gonadotropin; cAMP, adenosine 3′:5′-cyclic monophosphate; DbcAMP, N^6,O^2-dibutyryl adenosine 3′:5′-monophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GMP-P(NH)P, 5-guanylyl-imidediphosphate.

1) Testosterone production was studied by incubating the cells in buffer A in a Dubnoff shaker 2 hours at 33°C under O_2:CO_2 atmosphere (95:5). After incubation, the tubes were centrifuged (200 × *g* for 5 min) and testosterone in the supernatant was estimated by a specific radioimmunoassay (25).

2) The binding of (^{125}I)hCG was measured on interstitial cell particles. The isolated cells were homogenized in 10 mM Tris buffer pH 7.8 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM 1,4-dithioerythritol and 0.25 M sucrose (buffer B) using a Polytron PT 10 homogenizer. The particles were obtained by centrifuging the homogenate at 20,000 × *g* for 30 min. The pellet was resuspended in the same buffer and kept at −18°C until used.

Human chorionic gonadotrpin was labeled with ^{125}I by the lactoperoxidase method as previously described (26, 27). Specific activity was in the range of 100 μCi/μg. Binding was performed at two different dilutions of interstitial cell particles in the presence of saturating concentrations of (^{125}I)hCG (5×10^{-9} M). The incubation was performed in 0.3 ml of 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 Kalikrein units/ml (Zymophren, Specia, proteolytic enzyme inhibitor). After incubation at 37°C for 1 hour (equilibrium conditions) bound and unbound hormone were separated as previously described (19, 26). All binding experiments were performed in triplicate. Three additional samples were also run in the presence of a 100-fold excess of unlabeled hCG for estimation of non-specific binding.

To determine whether the apparent low levels of available receptor sites observed after hCG administration were due to occupancy or to actual loss of receptors, the binding of (^{125}I)hCG to interstitial cell particles was also measured in some experiments (Table 1) after homogenization of isolated interstitial cells in 4 M $MgCl_2$ as described by Chen *et al.* (28). This method dissociates the hCG-receptor complex without altering the binding capacity (28).

3) Adenylate cyclase was measured on interstitial cell particles as previously described (29), using 0.25 mM ATP for 10 min at 30°C. The enzyme activity was linear

TABLE 1

Binding of [^{125}I]hCG to rat interstitial cell particles homogenized either in buffer B or in 4 M $MgCl_2$

Rats (60 days old) were given a single injection of 500 IU of hCG and were killed at the times indicated. Interstitial cells were prepared as described under METHODS. Half of the cells of each group were homogenized in buffer B, the other half in 4 M $MgCl_2$, as described by Chen *et al.* (28). The [^{125}I]hCG bound to interstitial particles from control animals was 1.2 ± 0.2 pmoles/testis. The data represent the mean ± SD of specific binding determinations measured in triplicate and for three different concentrations of particles (50, 100 and 200 μg protein). ND = not detectable.

Hours after hCG injection	[^{125}I]hCG binding (% of control)	
	Buffer	4 M $MgCl_2$
Control	100 ± 5	98 ± 6
2 h	94 ± 6	112 ± 5
12 h	5 ± 0.2	12 ± 2
48 h	ND	ND
72 h	ND	ND
144 h	30 ± 5	28 ± 4

during the incubation time. Values expressed are averages of triplicate replications. Standard errors are less than 10% in all cases.

4) Cyclic 3′,5′-nucleotide phosphodiesterase was assayed in the same interstitial cell particles by the method described by Thompson *et al.* (30) using $2 \cdot 10^{-6}$ M [^{3}H]-cAMP substrate ($6 \cdot 10^4$ cpm per sample). For each experiment, the recovery of adenosine from the column was calculated using [^{14}C]adenosine and used for correcting each individual value (31). Results given represent averages of triplicate replication.

Testicular testosterone and cAMP content. Testes were removed, decapsulated and immediately immersed in 40 ml of ethanol at −60°C containing [^{3}H]cAMP (≃ 2000 cpm) and [^{3}H]testosterone (≃ 2000 cpm) and kept at this temperature for 4 hours. Thereafter the testes were homogenized in the same solvent. After centrifugation at 3000 × *g* for 10 min the supernatant was saved and the pellet extracted again by 30 ml of ethanol. The pool of supernatants was evaporated. Half of the extract was chromatographed on a Dowex 1-X2 column (0.6 × 6 cm, 200–400 mesh, Cl^- form) using the method described by Mao *et al.* (32) and the fraction containing the [^{3}H]cAMP was saved and evaporated. After acetylation (33) the cAMP was measured by radioimmunoassay (34). The testosterone content of the other half of the extract was measured by a specific radioimmunoassay (25). The last method was also used for determination of plasma testosterone.

Other methods. Protein was determined by the method of Lowry *et al.* (35) using bovine serum albumin as a standard, and DNA by the method of Burton (36).

RESULTS

In vivo refractoriness of Leydig cells after hCG injection. A single injection of 500 IU of hCG induced a sharp increase of plasma testosterone, the maximal values being reached two hours after injection (Fig. 1, left panel, continuous line). These results are similar to those reported in other studies (37, 38). Thereafter, levels decreased despite the fact that the concentration of plasma hCG remained high until 48 hours (19). A second peak of testosterone was observed between 60 and 98 hours. The pattern of testicular testosterone content following a single injection of hCG (Fig. 2, upper panel, continuous line) was very similar to that of plasma levels. It must be noted that the zenith of the second peak of testosterone in both plasma and testes varies from one experiment to another, but it has always been obtained (5 experiments) between 72 and 96 hours after hCG injection. Administration of hCG also induces within two hours a sharp increase on testicular cAMP content. Thereafter testicular content decreases but no second peak was observed (Fig. 2).

The *in vivo* refractoriness of Leydig cells between 12 and 60 hours after injection of 500 IU of hCG was proven by the fact that a second administration of hCG did not induce any significant increase either of plasma testosterone levels (Fig. 1, left panel, dotted line) or testicular testosterone and cAMP contents (Fig. 2, dotted lines). Steroidogenic responsiveness of Leydig cells to the second injection of hCG reappeared between 72 and 96 hours after first administration. Full steroidogenic responsiveness was recovered between 96 and 120 hours. However, testicular cAMP respon-

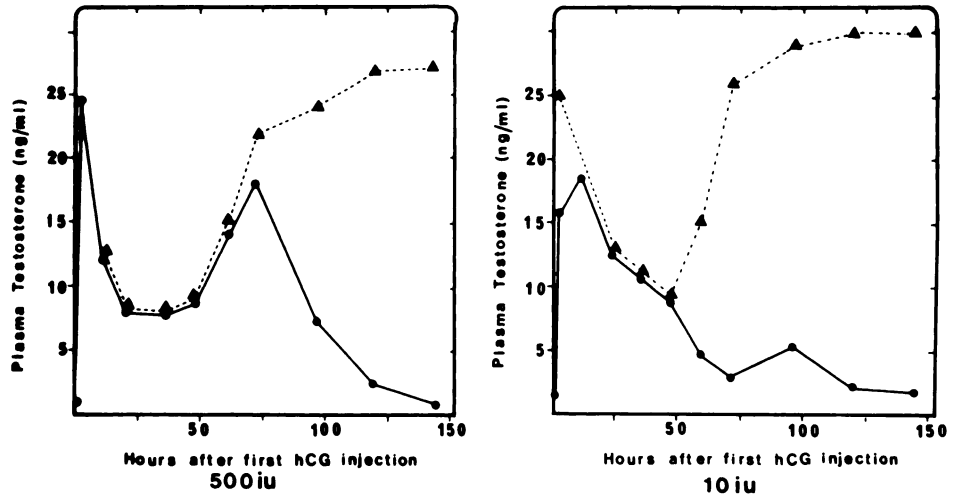

FIG. 1. *Effect of hCG on plasma testosterone*

Values shown represent mean values of three rats. At time 0, male rats 46 days old received i.m. injections of hCG 500 IU (left) or 10 IU (right). At the other times indicated, three rats were injected with saline (●) while another three rats were injected with 500 IU of hCG (▲). Animals of these subgroups were killed two hours later.

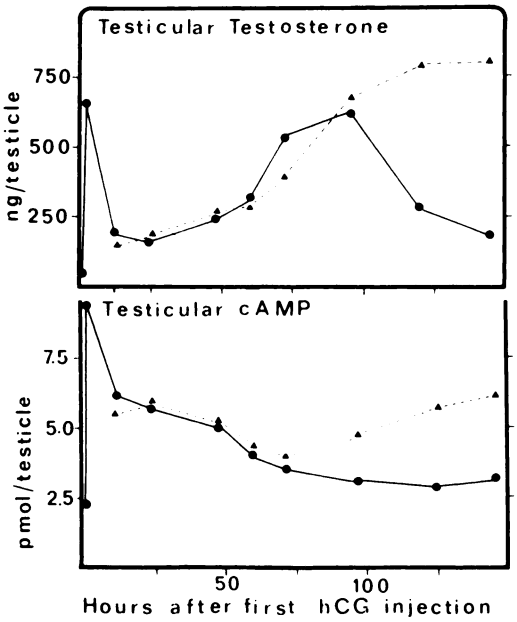

FIG. 2. *Effects of a single injection of 500 IU of hCG on testicular testosterone and cAMP contents*

siveness to the second injection of hCG was not completely recovered 144 hours after the first administration (Fig. 2).

After injection of 10 IU of hCG, maximum testosterone levels were reached between 6 and 9 hours. This peak was followed by a progressive decrease of plasma testosterone concentrations (Fig. 1, right panel). A second but smaller peak was observed around 72 hours.

The *in vivo* refractoriness of Leydig cells between 14 and 48 hours after injection of 10 IU of hCG was also demonstrated by the fact that a second administration of 500 IU of hCG did not induce any significant increase in either plasma testosterone levels (Fig. 1, right panel, dotted line) or testicular testosterone and cAMP contents (Fig. 2, upper and lower panels, dotted lines). Steroidogenic responsiveness of interstitial cells to a second injection of hCG appears between 48 and 60 hours after the initial administration (Fig. 1, right panel, dotted lines). Full responsiveness was recovered after 72 hours.

Human chorionic gonadotropin binding capacity and in vitro responsiveness of Leydig cells isolated from hCG-treated rats. The steroidogenic responsiveness of isolated Leydig cells from rats treated *in vivo* with 500 IU of hCG is shown in Fig. 3 (lower panel). Human chorionic gonadotropin and DbcAMP induced the same stimulation in cells from control animals. Two hours after hCG injection, the steroidogenesis of Leydig cells was six times higher and neither hCG nor DbcAMP was able to induce further stimulation. At 12 hours, tes-

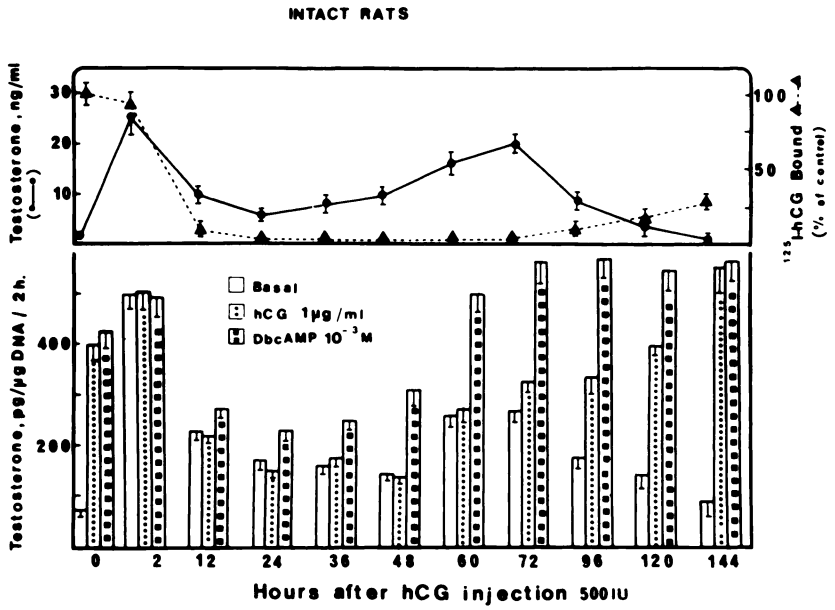

FIG. 3. *Effects of a single injection of 500 IU of hCG to 46-day-old male rats on plasma testosterone* (●) *and in vitro binding of [^{125}I]hCG by interstitial cell particles (upper panel)* (▲) *and in vitro testosterone production by isolated interstitial cells under several conditions (lower panel)*

The values given represent the mean ± SD of triplicates of two separate experiments (pool of three rats for each determination). The [^{125}I]hCG bound to interstitial particles from control animals was 0.6 ± 0.2 pmol/mg DNA.

tosterone production was lower and cells did not respond to hCG, while DbcAMP induced a lower but significant stimulation. Starting at 36 hours, responsiveness to DbcAMP progressively recovered toward control levels. At 72 hours complete response to DbcAMP was reached, while refractoriness to hCG persisted. Full responsiveness to hCG was removered only at 144 hours.

The apparent number of hCG binding sites in testis was not significantly reduced two hours after injection of 500 IU of hCG (Fig. 3, upper panel). At this time, however, plasma hCG (300 ng/ml) and testosterone levels were very high (19). Thereafter, the binding capacity declined, and by the 12th hour it reached 10% that of controls. It remained at undetectable levels until 72 hours. This decrease in binding capacity was mainly due to receptor loss and not to receptor occupancy since similar results (Table 1) were obtained with interstitial cell particles prepared by the method described by Chen *et al.* (28). Apparent hCG receptors progressively recovered but they were still lower than in control animals six days after hCG injection.

After injection of 10 IU of hCG the decrease of hCG binding capacity of testicle particles was less marked than that observed after administration of 500 IU. The lowest values were observed between 36 and 60 hours after administration; hCG binding capacity then progressively recovered toward control levels (between 120 and 144 hours) (Fig. 4, upper panel).

Interstitial cell preparations (Fig. 4, lower panel) showed that one hour after injection of 10 IU of hCG testosterone production was stimulated by both hCG and DbcAMP. Between 12 and 48 hours after administration the sensitivity of the cells to stimulation by DbcAMP was decreased, while sensitivity to hCG stimulation was lost. Similarly, after injection of 500 IU of hCG, responsiveness to DbcAMP was recovered earlier than responsiveness to hCG. Two findings are noteworthy: first, when total refractoriness to hCG was observed between 12 and 48 hours, the number of receptors was about 30% of control; second,

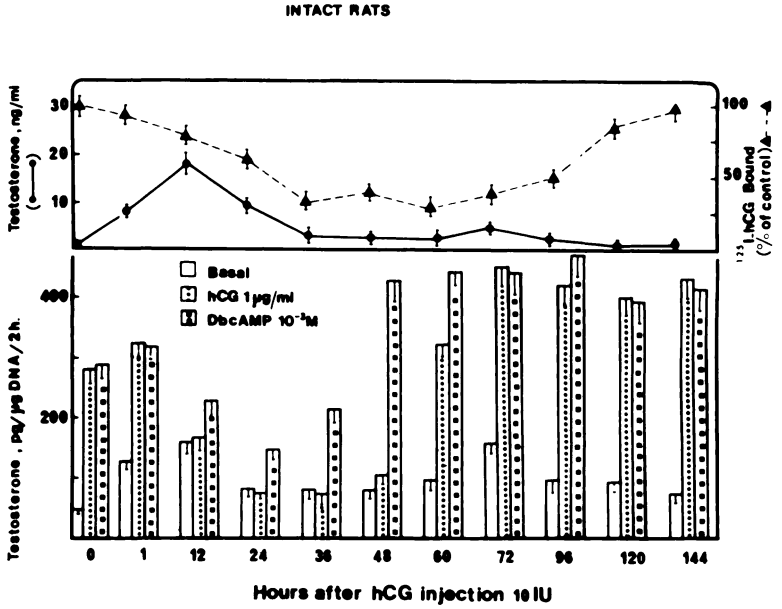

FIG. 4. *Effects of a single injection of 10 IU of hCG on testosterone levels in 46-day-old male rats*

the number of receptors at 72 and at 96 hours—when the recovery of steroidogenic responsiveness was complete—was similar to the number observed between 24 and 48 hours.

Adenylate cyclase and cAMP phosphodiesterase activities after hCG injection. Adenylate cyclase was measured in either freshly prepared or liquid nitrogen-stored interstitial cell particles. In preliminary experiments in control animals, maximal stimulation was obtained with 10 mM NaF and 10^{-4} M GMP-P(NH)P. On the other hand, under our experimental conditions, the stimulation produced by hCG (10^{-9} to 10^{-6} M) was very small (less than 20% over the baseline) and inconsistent. Using the same method we have found good stimulation of adenylate cyclase of ovarian particles by hCG (29).

Basal, NaF, and guanyl nucleotide-stimulated adenylate cyclase activities following injection of 500 IU of hCG are shown in Fig. 5 (lower panel). Only the basal activity two hours after injection of hCG was higher than that of controls. At all other times guanyl nucleotide and NaF-stimulated adenylate cyclase activities were not significantly different from those of controls. Due to the inconsistent and insignificant hCG stimulation, the results are not shown.

cAMP phosphodiesterase activity (Fig. 5, upper panel) was similar in controls and following hCG administration.

DISCUSSION

This study confirms that hCG exerts a negative control on its own membrane-bound receptor (17–22), and this effect is dose-dependent (19, 41). Decreased binding capacity cannot be explained simply by occupancy of the binding sites by the injected hCG (21 and Table 1). It could be due either to inactivation or degradation of the receptor by the bound hormone, or to an active process (increased degradation or decreased synthesis of the receptor) initiated by the hormone-receptor interactions.

Whatever the exact mechanism responbile for this receptor "down-regulation," it seems clear that it cannot entirely explain the refractoriness of Leydig cells. First, Leydig cells very soon become refractory to hCG stimulation, at the time when they still have "free" apparent receptors (Fig. 4) and when steroidogenesis is normally stimulated by DbcAMP. Since the catalytic subunit of the receptor-adenylate cyclase complex was not affected (Fig. 5), the most likely explanation for this paradox may be

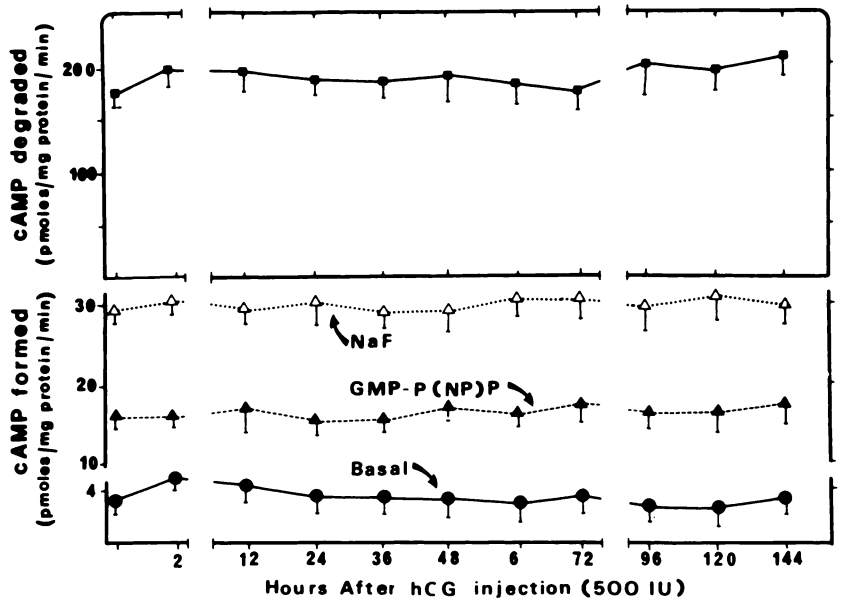

FIG. 5. *Effects of a single injection of 500 IU of hCG to 50-day-old male rats on in vitro phosphodiesterase (upper panel) and adenylate cyclase activity (lower panel) of interstitial cell particles*
Basal (●), GMP-P(NH)P 10^{-4} M (▲), NaF 10 mM (▲).

that there is a defective coupling between the receptor and the adenylate cyclase (42). This hypothesis has already been proposed by Bockaert *et al.* (11) to explain the *in vivo* desensitization of Graafian follicle membrane by LH, and it also probably explains the refractoriness of thyroid cells to TSH (9) and of adrenal cells to ACTH (39).

Second, between 12 and 48 hours after injection of 10 IU of hCG, and between 12 and 60 hours after injection of 500 IU of hCG, Leydig cells presented complete refractoriness to hCG and partial refractoriness to DbcAMP. This phenomenon, Leydig cells refractoriness to hCG and partial response to DbcAMP, has been reported by Sharpe (22) following administration of small doses of hCG to immature rats. These results suggest that, in addition to the membrane receptor abnormality, some undefined intracellular step beyond cAMP formation might be altered in these cells. However, their steroidogenic activity, as revealed by plasma testosterone levels and Leydig cell testosterone levels and Leydig cell testosterone production, continued above control levels even though further stimulation was blocked.

A third possible mechanism for refractoriness of Leydig cells to hCG is the induction of increased phosphodiesterase activity, as has been shown in other systems (6, 7). This hypothesis seems unlikely since, as indicated, all cell preparation studies were performed in the presence of the potent phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, and the interstitial cell phosphodiesterase activity of hCG-treated rats was similar to that of untreated rats (Fig. 4).

It also seems unlikely that testosterone itself would be responsible for the refractoriness to hCG stimulation, since high doses of this steroid administered i.m. produce a 20 to 100-fold increase of plasma levels but do not modify either the binding capacity or the Leydig cell responsiveness to hCG or DbcAMP (42).

The time course of the resensitization of interstitial cells to DbcAMP and to hCG was different. Full responsiveness to DbcAMP was reached 48 to 72 hours before that to hCG. This suggests that the induced modification of the intracellular steroidogenic step was overcome earlier than that of the receptor. In addition, full steroidogenic responsiveness to hCG, both *in vivo*

and in isolated cell preparations, preceded the recovery of the total number of binding sites of this hormone. In this respect it is interesting to note the apparent absence of hCG binding sites at 72 hours (Table 1) while steroidogenic responsiveness to hCG (Figs. 1 and 3) had partially recovered. These results suggest a recovery of the proposed abnormality of the coupling system and imply that only a small number of binding sites, not detectable by binding studies, are necessary to obtain stimulation of testosterone production. This has been demonstrated by Catt *et al.* (40) in Leydig cells that were not pre-stimulated. On the other hand, full cAMP responsiveness to the second injection of hCG was still not completely recovered six days after the first injection of 500 IU of hCG. This could indicate that a complete recovery of the number of binding sites might be necessary to obtain maximal cAMP formation.

It is noteworthy that during recovery from hCG-induced refractoriness, the response to DbcAMP and, later, to hCG exceeded the control values by approximately 50% (Fig. 3, lower panel). The most likely explanation for this overshoot is the maturation of the rat testis during the time of the experiment. All the experiments were started when the rats were 46 to 52 days old and ended when they were 52 to 58 days old.

In sum, the present study has shown that at least two mechanisms are involved in the hCG-induced refractoriness of Leydig cell steroidogenesis: an abnormality of some step of steroidogenesis beyond cAMP and a decrease in the number of hCG binding sites. However more studies are needed to elucidate the sequence and the molecular mechanism involved in these phenomena.

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