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Inhibition of ^{125}I-Labeled Human Chorionic Gonadotropin Binding to Gonadal Receptors by a Factor Obtained From Rat Testicular Tissue

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ABSTRACT

The ability of a factor obtained from a 105,000 × g supernatant of rat testis to inhibit the hCG binding to gonadal receptors was studied. This factor was partially heat stable, not steroid in nature, and presented a molecular weight under 12,000. Binding studies indicated that the hCG binding inhibitor interfered with the formation of the hormone-receptor complex mainly by competing with hCG for the same binding sites. Consequently, it was demonstrated that the inhibitor prevented the interaction of hCG and its receptor in both testicular and ovarian tissues. Under in vitro binding conditions, it was observed that as the quantities of the inhibitor increased during the first incubation with testicular homogenate, and after removing the inhibitor and washing the pellet, less and less of the added labeled hCG was bound during the second incubation step. On the other hand, the hormone already bound to the tissue during the first incubation could not be displaced by increasing amounts of the inhibitor in the second incubation. Thus, binding of the inhibitor, or hormone, to the tissue in the first step was not readily exchangeable with the subsequent inhibitor, or hormone, added in the second step. These findings were consistent with the view that binding to LH/hCG receptors may proceed in an irreversible manner.

Although the hCG binding inhibitory activity was detected in many of the tissue extracts and in serum of the rat, only testicular extracts showed the ability to retain a significant part of this activity (∿40%) after heating for 30 min in a boiling water bath, under equivalent experimental conditions. The content of hCG binding inhibitor in the testis was also examined during the gonadotropin-induced reduction in the availability of LH/hCG receptors following in vivo administration of 100 IU of hCG. Results indicated that the receptor depletion and replenishment processes proceeded without significant changes in the hCG binding inhibitory activity in the testis. It was concluded that the phenomenon of down-regulation of LH/hCG receptors and the quantity of testicular inhibitor were not necessarily related to one another.

INTRODUCTION

Recent studies have described several low molecular weight substances capable of inhibiting gonadotropin binding to target tissue. Leindenberger et al. (1976) have reported a material of about M_r 2000 obtained from human serum which inhibited binding of luteinizing hormone (LH) to testicular receptors and had LH-like activity ("mini LH"). The presence of factors which interfere with the interaction of follicle-stimulating hormone (FSH) and its receptors has been demonstrated in rat testis (Reichert and Abou-Issa, 1977), in human serum (Reichert et al., 1979), and in follicular fluid derived from bovine follicles (Darga and Reichert, 1978). Yang et al. (1976a, b) reported a low (M_r 3800) molecular weight inhibitor of LH receptor binding in extracts prepared from ovaries of pregnant and pseudopregnant rats, but not from the ovaries of mature nonpregnant rats or immature rats, nor was it found in testis extracts. A similar inhibitor, present in an extract of pig corpus luteum, has also been reported to diminish human chorionic gonadotropin (hCG) binding to porcine granulosa cells (Sakai et al., 1977). Factors with the ability to inhibit the binding of gonadotropins to gonadal receptors may eventually be found to play an important role in the control and mechanism of action of these hormones. In this report, we describe the presence

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of an hCG binding inhibitor in rat testis, which competes for the LH/hCG binding sites on testicular tissue and which can also inhibit the binding of ^{125}I-labeled hCG to ovarian receptors. We also examine whether the content of hCG binding inhibitor in the testis of hCG-treated rats is correlated with the gonadotropin-induced reduction in LH/hCG receptor availability.

MATERIALS AND METHODS

Rats of the Sprague-Dawley strain were utilized. They were housed in a 12L:12D environment and fed rat chow ad libitum. The rats were sacrificed by decapitation immediately prior to use. Chloramine T was purchased from the Eastman Kodak Co.; egg albumin, crystallized twice, from Schwartz-Mann; sodium metabisulfite from Malinchrodt Chemical Works; carrier-free Na^{125}I from New England Nuclear; and hCG (10,000 IU/ampoule) from Ayerst Labs. Other chemicals were of reagent or analytical grade.

Iodination Procedure

A highly purified hCG (CR 121; 13,450 IU/mg), a gift from Dr. V. K. Bhalla, was iodinated by the chloromine T method, modified to allow the retention of biologic activity (Reichert and Bhalla, 1974; Chen et al., 1979). The specific activity of the ^{125}I-hCG ranged from 15–18 μCi/μg. The labeled hormone was diluted with a 0.01 M phosphate buffer (pH 7.5) containing 5 mM $MgCl_2$, 0.1 M sucrose, and 0.1% (w/v) egg albumin (henceforth referred to as the albumin/phosphate buffer) to a final concentration of 2.5 ng/50 μl of solution (7.5×10^{-14} M), and stored frozen in several vials.

Preparation of the Tissue Extracts for Inhibition Studies

Testicular extract. The hCG binding inhibitory factor present in testicular extracts was obtained essentially as described by Reichert and Abou-Issa (1977) for the preparation of FSH binding inhibitor. Testes from mature rats (200–300 g BW) were decapsulated and homogenized in a Teflon pestle tissue grinder at a concentration of 1 g tissue per milliliter of albumin/phosphate buffer. The homogenate was strained through a single layer of cheesecloth and centrifuged at 12,000 × g for 60 min at 4°C. The supernatant was then centrifuged at 105,000 × g for 60 min. After each centrifugation a small lipid layer was removed with a Pasteur pipette. The resulting supernatant was used for experiments on hCG binding inhibitory activity.

Other tissue extracts. A similar procedure was used to examine the presence of hCG binding inhibitor in other tissue extracts such as liver, kidney, and brain. To prepare ovarian extracts from mature and pregnant rats, the tissue was homogenized at a ratio of 1 g tissue per 2 ml of albumin/phosphate buffer.

Preparation of Testicular Receptors and Binding Assays

Rat testicular particulate receptors were prepared as described elsewhere (Rojas and Bhalla, 1979; Bhalla et al., 1979). Briefly, testes from mature male rats were decapsulated and homogenized at a ratio of 2 ml albumin/phosphate buffer per gram of tissue. The homogenate was filtered through a single layer of cheesecloth, then centrifuged at 1500 × g for 10 min at 4°C, and the supernatant discarded. The pellet was centrifuged at 20,000 × g for 10 min at 4°C to expel the excess buffer and resuspended in fresh albumin/phosphate buffer to a final concentration of 1 g pellet per 10 ml of buffer. Binding studies were performed using this particulate receptor preparation or testicular homogenate. The standard binding assays consisted of the incubation of 1.25 ng of ^{125}I-hCG (25 μl) and 20 mg wet weight of testicular homogenate in the absence or presence of an aliquot of tissue extract. The final reaction volume was 1 ml in albumin/phosphate buffer. The mixture was incubated at 37°C for 2 h in a metabolic shaking bath. At the end of the incubation period, the tubes were centrifuged at 1500 × g for 10 min at 4°C, and the supernatant containing the free hormone was decanted. The tubes containing tissue pellets were inverted over adsorbent paper in a rack and kept at 4°C for 30 min prior to being counted for radioactivity in a gamma counter. The data were also analyzed by subtraction of nonspecific

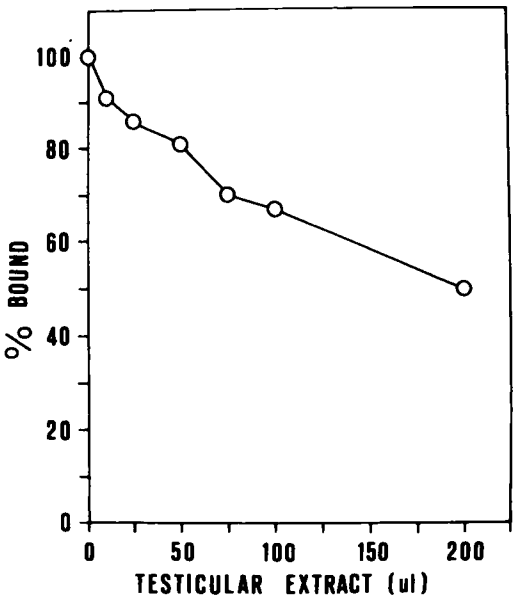

FIG. 1. Dose-response effect of the testicular extract on the inhibition of specific hCG binding to rat testis. The receptor preparation (20 mg, wet tissue weight) was incubated with ^{125}I-hCG (1.25 ng) in the presence of 0, 10, 25, 50, 75, 100, and 200 μl of testicular extract. The incubation was carried out in a final volume of 1 ml at 37°C for 2 h as described in Materials and Methods. The 100% control values represent specific binding of ^{125}I-hCG to testicular homogenate in the absence of tissue extract (23.8% of total amount of tracer hormone added). Data are the mean of duplicate determinations.

binding according to the method described elsewhere (Rojas and Bhalla, 1979; Chen et al., 1979). Nonspecific binding was less than 5% of the total binding. For inhibition kinetics studies, the testicular homogenate was incubated with ^{125}I-hCG under the conditions described above and with increasing concentration of unlabeled homologous hCG (0–100 ng) in the absence or presence of a fixed amount of either direct testicular extract or extract heated for 30 min in a boiling bath. The data were then analyzed by a double reciprocal plot according to procedures described elsewhere (Rojas and Bhalla, 1979; Chen et al., 1979; Tsuruhara et al., 1977; Hsueh et al., 1977).

Preparation of Ovarian Receptors and Binding Assays

Preparation of 2000 × g ovarian homogenates was largely carried out as described by Lee and Ryan (1973). Ovaries from mature female rats were sliced, and a 1:10 (w/v) tissue homogenate was prepared in cold albumin/phosphate buffer using 10 strokes of a glass Teflon homogenizer. The homogenate was strained through two thin layers of cheesecloth and centrifuged at 2000 × g for 15 min at 4°C. The pellet was centrifuged at 20,000 × g for 10 min at 4°C to expel the excess buffer, and resuspended at the equivalence of 100 mg ovary per milliliter albumin/phosphate buffer. For binding assay, 100 μl of the 2000 × g pellet of ovarian homogenate were incubated with 1.25 ng ^{125}I-hCG (25 μl) and an aliquot of the testicular extract or buffer. The final volume was 1 ml in albumin/phosphate buffer. In some of the tubes, 500-fold excess of unlabeled hCG was added to determine nonspecific binding. Incubation was carried out at 37°C for 2 h after which the tubes were chilled and immediately centrifuged at 2000 × g for 15 min at 4°C. The tubes were then processed for radioactivity count as described above.

In vivo Administration of hCG and Determination of Gonadotropin Binding Sites

The schedule by which hCG was administered has been described by Rojas and Bhalla (1979). Briefly, mature male rats, divided into several groups (7 rats/group), received one i.p. injection (100 IU) of hCG (Ayerst Labs.) at 0900 h. The rats were sacrificed by groups on Days 1, 2, 3, 5, and 7 after the injection; the testes were removed for binding studies and for determination of the hCG binding inhibitory activity. Control animals received injections of saline. For each data point, a single homogenate prepared from three rats was taken for binding assays, and one prepared from four animals was taken for measurement of the inhibitory activity. The determination of binding sites was achieved through displacement experiments generated in the presence of 0–1000 ng of unlabeled hCG and by using 20 mg testicular homogenate and 1.25 ng of ^{125}I-hCG. The final reaction volume was 1 ml and incubation was carried out at 37°C for 2 h. The binding data were then analyzed by Scatchard analysis according to the methods generally used (Rojas and Bhalla, 1979; Chen et al., 1979; Tsuruhara et al., 1977; Hsueh et al., 1977). The apparent number of binding sites (B_{max} values) was calculated from the linear regression analysis performed on the lines obtained from the Scatchard plots. The relative quality of the data (P values) was assessed by applying Student's t test and by considering the significance of parameters derived from each regression (Rojas and Bhalla, 1979). The determination of the hCG binding inhibitory activity was carried out by using 200 μl of tissue extract in the standard binding assay. Replicated values were obtained in all cases.

Other Procedures

Rat serum was obtained by allowing blood to clot and centrifuging at 5000 × g for 30 min to remove the insoluble components.

The stability of the hCG binding inhibitor to heat was assessed by dividing a sample into two aliquots. One aliquot was left untreated, and the other was heated in a boiling water bath for 30 min. After heating, the aliquot was centrifuged at ∿10,000 × g for 10 min and the clear supernatant was used in binding assays.

To assess whether the testicular factor was steroid in nature, diethyl ether extraction and adsorption to charcoal were carried out as described by Darga and Reichert (1978). Accordingly, aliquots of the extract were treated with diethyl ether at room temperature at a ratio of one volume of the supernatant per 3 volumes of diethyl ether for two consecutive extractions. Adsorption by charcoal was performed at a proportion of 60 mg charcoal per 2 ml extract and incubated for 1 h at 4°C. The fractions obtained after treatment were tested for residual hCG binding inhibitory activity in the standard binding assay.

RESULTS

Effect of the Quantity of the Testicular Extract on the Inhibition of Specific hCG Binding to Rat Testis

The dose-response relationship between the quantity of the testicular extract and the degree of inhibition of ^{125}I-hCG binding to testicular LH/hCG binding sites is shown in Fig. 1. In the presence of 200 μl of tissue extract, ∿50% of the specific binding was inhibited compared with controls. Responses for maximal binding inhibition have not yet been determined.

Some Properties of the bCG Binding Inhibitor

Dialysis of the testicular extracts against 20 times the volume of distilled water at 4°C for 72 h with frequent change of water and vigorous stirring resulted in ∿90% loss in inhibitory activity of the nondialyzable portion (Fig. 2). More extensive dialysis did not completely reduce the inhibitory activity of the extract. The dialyzer tubing used (3787-D12, Arthur H. Thomas Co.) was reported by the manufacturers to have a retention of M_r 12,000 and

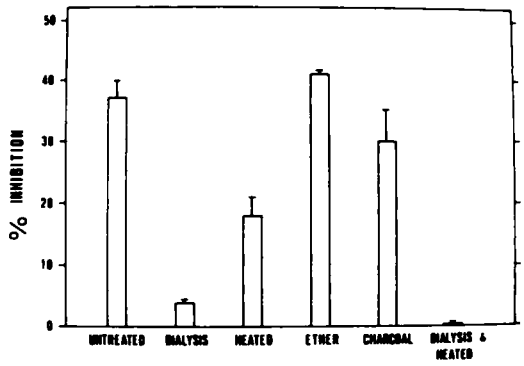

FIG. 2. Effect of various treatments on hCG binding inhibitory activity of testicular extract. The fractions, obtained after the different treatments, were tested for residual hCG binding inhibitory activity in the standard binding assay. Data are expressed as a percentage of binding inhibition in the presence of 200 μl of tissue supernatant. Each value represents the mean ± SEM of three separate determinations.

higher. The hCG binding inhibitory activity remaining in the nondialyzable portion of the extract was completely destroyed after heating for 30 min in a boiling water bath (Fig. 2). When the dialysis was carried out against a single batch of water (the ratio of extract to water was 1:20) and the dialysate was lyophilized and reconstituted to half the original volume with albumin/phosphate buffer, 200 μl of the reconstituted material inhibited ∼30% of the hCG specific binding, indicating that the inhibitor could pass through the membrane.

The incubation of the original testicular extract in a 100°C bath for 30 min resulted in a loss of ∼50–60% of inhibitory activity (Fig. 2), precluding the possibility that all of the inhibitory activity detected in the direct extract was due to endogenous LH. Furthermore, the heated fraction showed no appreciable effect on a radioimmunoassay for rat luteinizing hormone (data not shown).

Adsorption of the tissue extract by charcoal or extraction with diethyl ether did not result in significant loss of inhibitory activity, indicating that the hCG binding inhibitor does not seem to be steroid in nature (Fig. 2). No detectable change of the inhibitory activity was observed when the testicular extracts were kept in liquid nitrogen for at least 3 months. Therefore, either a freshly prepared supernatant or a supernatant previously stored in liquid nitrogen was used in subsequent studies.

Inhibition Kinetics Studies and Effects of Incubation Steps on hCG Binding Inhibitory Activity

Evidence that the hCG binding inhibitor was effective in preventing formation of the hormone-receptor complex is shown in Table 1. Because a similar pattern of results was obtained by using direct testicular extract, or tissue extract heated for 30 min in a boiling water bath, only the latter is shown. In this experiment, the testicular homogenate was first incubated with the inhibitor factor, after which the inhibitor was removed and the pellet washed with the assay buffer. The homogenate

TABLE 1. Effects of hCG binding inhibitor on the formation of hormone-receptor complex.

Assay	First incubation mixture[a]	Additives in the second incubation mixture	Counts bound	% Decrease in counts bound vs control
1	Homogenate	^{125}I-hCG	5145 ± 48	Control
2	Homogenate + heat-treated testicular extract	^{125}I-hCG	3215 ± 81	38[b]

[a]First and second incubation mixtures were both carried out at 37°C for 2 h in a final volume of 1 ml with albumin/phosphate buffer. One hundred microliters of testicular homogenate (10 mg wet tissue) were incubated with 400 μl of a heated (30 min in a 100°C bath) tissue extract. After the first incubation, tubes were centrifuged and the pellets were decanted and washed with 1 ml of cold albumin/phosphate buffer. The pellets were then reconstituted and incubated a second time with 1.25 ng ^{125}I-hCG. The tubes were centrifuged and the pellets counted for radioactivity as described in Materials and Methods. Each value represents the mean ± SEM of three separate determinations.

[b]$P<0.005$, based on Student's t test for nonpaired variates.

was then reconstituted and incubated with ^{125}I-hCG. The results indicate that preincubation with the testicular factor significantly diminished the subsequent binding of ^{125}I-hCG to testicular binding sites (38%; $P<0.005$) compared with control experiments where the first incubation of the receptor preparation was with buffer alone. The use of heat-treated testicular supernatant precluded the likelihood that the effects described in Table 1 were attributable to degradative enzymes, and revealed that the inhibitor present in the original testicular extract retained the ability to prevent the formation of the hormone-receptor complex after heating.

In view of the experimental results indicating that the testicular factor inhibited the formation of the hormone-receptor complex, it was pertinent to see whether the inhibition was due to competition between the inhibitor and ^{125}I-hCG for the same binding site, or to competition for different sites, with the binding of the inhibitor preventing the binding of ^{125}I-hCG to its receptor. The inhibition kinetics study by Lineweaver-Burk analysis of the hCG binding inhibitory activity in the direct testicular extract and in a tissue extract heated for 30 min in a boiling water bath is illustrated in Fig. 3. The testicular receptors were incubated with a fixed concentration of ^{125}I-hCG and with increasing concentrations of unlabeled hCG in the absence or presence of a fixed amount of testicular factor as described in Materials and Methods. The results showed a competitive type of inhibition with both testicular supernatants assessed, demonstrating that the hCG binding inhibitor bound to the receptor itself.

To investigate further the nature of the binding between the inhibitor factor to testicular receptors, two additional experiments were undertaken. In one experiment, testicular homogenate was preincubated with increasing amounts of original tissue extract (0–200 μl). The inhibitor was then removed, and the pellets were reconstituted with buffer and incubated with 1.25 ng of ^{125}I-hCG (Fig. 4A, Line A). In the other experiment, testicular homogenate was first incubated with ^{125}I-hCG. After incubation, the unbound hormone was removed, the pellet reconstituted, and then incubated with increasing amounts of testicular extract (0–200 μl). No labeled hormone was added in the second incubation (Fig. 4A, Line B). Results from the first experiment (Fig. 4A, Line A) show that as the quantities of testicular inhibitor increased during the preincubation of the tissue, progressively less of the subsequent labeled hormone was bound in the second incubation step. On the other hand, Line B of Fig. 4A shows that the hormone already bound to the tissue in the preincubation could not be displaced as a function of increasing quantities of the testicular factor during the second incubation. It also became evident from this experiment that the inhibitor, at the concentration assessed, had only a slight effect on dissociation of the preformed hormone-receptor complex. These observations indicate that, on the one hand, the inhibitor and the hormone bind to the same receptor

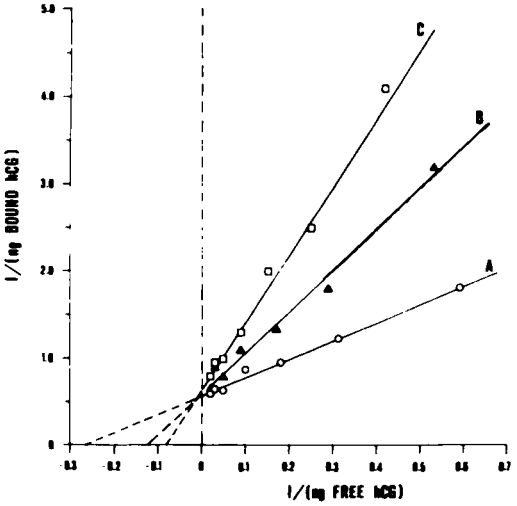

FIG. 3. Double reciprocal plots of bound ^{125}I-hCG vs free ^{125}I-hCG at fixed levels of testicular inhibitor. A) in the absence of inhibitor; B) 400 μl of a heated testicular extract (30 min in a 100°C bath); and C) 200 μl of direct testicular extract. Rat testicular homogenate (20 mg) was incubated with ^{125}I-hCG (1.25 ng) and with the indicated fixed amounts of testicular supernatant in the presence of 0–100 ng of unlabeled hCG. The tubes were incubated for 2 h at 37°C and then processed as described in Materials and Methods. The data obtained were converted from cpm to nanograms of hormone bound (and/or free) through the dilution of specific activity method and plotted according to Lineweaver-Burk equation. The K_d value for hormone binding in the absence of inhibitor was 1.15×10^{-10} M. Molecular weight of hCG was taken as 33,000. The regression for curve A was $y = 2.13x + 0.562$; for curve B, $y = 4.79x + 0.585$; and for curve C, $y = 8.18x + 0.642$. The correlation coefficient (r) for the lines was 0.99 in all cases. Scatchard plots of the data yielded similar binding parameter values. All values in the graph represented the mean of duplicate determinations.

binding sites (see above) and, on the other hand, the binding of the inhibitor or hormone to the tissue during the first incubation was not readily exchangeable with the binding of the inhibitor or the hormone added in the second incubation. When the same experiments were repeated using unlabeled hCG instead of testicular inhibitor, a similar pattern of binding behavior was observed, i.e., the unlabeled hCG was unable to displace the earlier bound ^{125}I-hCG, and less labeled hCG was bound to the tissue after preincubation with increasing amounts of unlabeled hormone (Fig. 4B). These findings confirmed a previous report by Chen et al. (1979) who demonstrated the irreversibility of hCG and LH binding to testicular homogenates by performing the same experimental approaches as shown in Fig. 4B. Taken together, our data therefore were also consistent with the view that binding of inhibitor to LH/hCG receptors may proceed in an irreversible manner.

Inhibition of hCG Binding to Ovarian Receptors by Testicular Extracts

If the testicular inhibitor was able to bind the hCG testicular receptor itself, as shown earlier (Fig. 3), it would then be expected that the inhibitor also binds receptors of ovarian tissue. As demonstrated in Fig. 5, this was indeed the case. ^{125}I-hCG binding to ovarian receptors obtained from a 2000 × g homogenate was significantly inhibited by testicular extract in a dose-response relationship, with 200 μl of tissue extract reducing binding of 1.25 ng ^{125}I-hCG by 40% compared with control assays. When testicular supernatant was heated in a boiling water bath for 30 min, 200 μl of the remaining supernatant was able to inhibit the binding of ^{125}I-hCG to ovarian tissue by 15% (data not shown).

hCG Binding Inhibitory Activity During hCG-Induced Changes in Testicular Receptors

To obtain information on the possible relationship between the hCG binding inhibitor and the gonadotropin-induced reduction in the availability of LH/hCG receptors, we examined the inhibitory activity in the testis during the down-regulation of the LH/hCG receptors following in vivo administration of hCG. As shown in Fig. 6, 24 h after the injection of rats with 100 IU of hCG, the number of LH/hCG-specific binding sites was undetectable. On Day 3, the sites began to increase and return to normal by Day 7. This response was very similar to that reported earlier (Rojas and Bhalla, 1979; Saez et al., 1978; Hsueh et al., 1977; Catt et al., 1980). In contrast to this pattern, the hCG binding inhibitory activity in the testis of hCG-treated rats did not significantly change during the entire receptor depletion and replenishment processes (Fig. 6). After heating of the extracts for 30 min in a 100°C bath, no changes in the inhibitory activity were observed in any of the different groups (data not shown). It appears, therefore, that there is no apparent correlation between the testicular inhibitory activity and the down-regulation of LH/hCG receptors in rat testis.

hCG Binding Inhibitory Activity in Other Tissues and Serum

The testing of inhibitory activity in other tissues and serum from the rat is summarized in Table 2. In all cases, 100 mg tissue equivalent of extracts, or 100 μl of serum, showed appreciable inhibition of ^{125}I-hCG binding to testicular receptors. However, such activity was completely lost in most of these tissue samples after heating in a boiling water bath for 30 min, with the exception of the extract obtained from testicular tissue, which retained ∼40% of its capability to inhibit hCG binding despite the drastic treatment (see above). In samples showing nondetectable activity after heating, the increase up to 200 mg tissue equivalent (extracts), or 200 μl (serum), of the heat-treated material also failed to demonstrate significant inhibitory capacity. Higher doses were not tested. For the testicular extract, the multiple-dose assays (10–100 μl) revealed a parallel response when heat-treated extracts and untreated control were compared for inhibitory activity (not shown). As indicated in Table 2, extracts of ovaries from pregnant rats and serum of intact male rats could retain, at a lesser extent, some inhibitory activity after heating, but analysis of variance indicated no significant difference ($P>0.05$) of these values from the specific ^{125}I-hCG binding in the presence of control buffer alone.

DISCUSSION

In this report we have demonstrated the presence of a factor obtained from rat testis having the ability to inhibit hCG binding to gonadal receptors. The factor was partially heat

stable, not steroid in nature, and with M_r <12,000, indicating that some of its physicochemical properties might be similar to those reported for other inhibitors of gonadotropin binding, such as a LH-receptor binding inhibitor described by Yang et al. (1976a) and the FSH binding inhibitors described by Reichert et al. (1979), Darga and Reichert (1978), and Reichert and Abou-Issa (1977). The testicular inhibitor, on the other hand, was clearly different from the hCG binding inhibitors present in other tissue extracts and serum of the rat (Table 2) in that the latter lost all their inhibitory activity by heating, while the testicular factor retained ∿40% of it under equivalent experimental conditions.

Binding studies indicated that the LH/hCG binding inhibitor prevents hCG binding mainly, if not entirely, by interfering with the formation of the hCG-receptor complex. Also, inhibition kinetics studies performed with the direct testicular extract or with a heat fraction, showed that the inhibitor competes with hCG for the same binding sites, and consequently, was able to prevent the interaction of hCG and its receptor in testis and ovary. This was in great contrast to a LH binding inhibiting factor found in ovaries of pregnant or pseudopregnant rats (currently known as LHRBI), which was reported to inhibit LH binding to ovarian LH receptors, and did not inhibit LH binding to testicular LH receptors (Yang et al., 1976b). Such observations have led the authors to believe that ovarian LH receptor may be structurally different from testicular receptor (Yang et al., 1976b). It appears, therefore, that the LHRBI and the hCG binding inhibitor isolated from testis are distinct molecular entities with different mechanisms of action. Also, the hCG binding inhibitor cannot be identical to gonadotropin-binding factors extracted from rat testis by ethanol (Bhalla et al., 1976) because these ethanol-soluble factors were demonstrated to be remarkably heat-resistant and to have a very

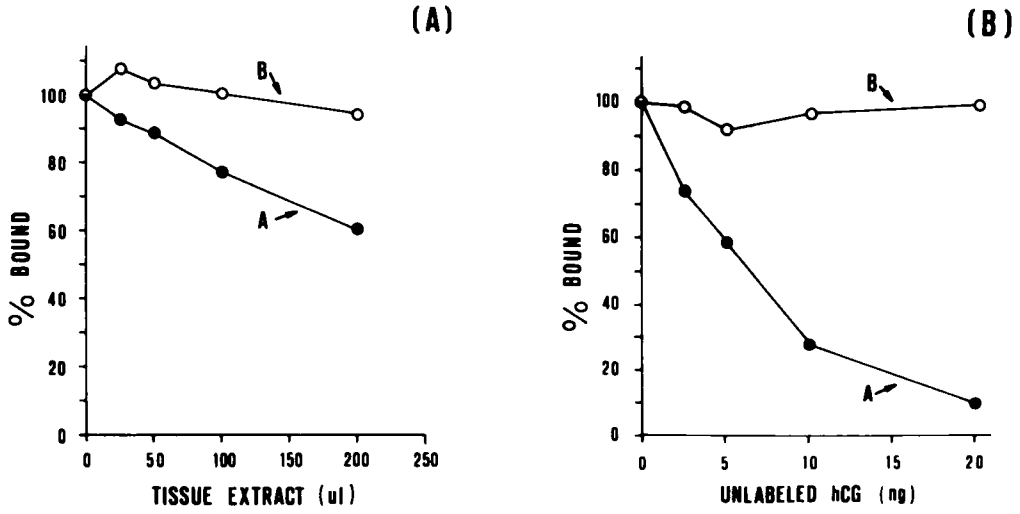

FIG. 4. A) Binding of ^{125}I-hCG to testicular homogenate pretreated either with increasing quantities of testicular extract (0–200 μl) or with 1.25 ng of ^{125}I-hCG. Testicular homogenate (20 mg) was incubated with the various amounts of testicular extract or with the constant amount of ^{125}I-hCG at 37°C for 1 h. After the incubation, free inhibitor, or hormone, was removed; the pellets pretreated with inhibitor were incubated with 1.25 ng of ^{125}I-hCG, and the pellets pretreated with ^{125}I-hCG were incubated with increasing quantities (0, 25, 50, 100, and 200 μl) of testicular extract. Albumun/phosphate buffer was used to bring the volume of the reaction mixture up to 1 ml. The tubes were incubated again at 37°C for 1 h and then processed as described in Materials and Methods. Line A shows the competitive inhibition plot of the binding data obtained using tissue pretreated with the inhibitor, and Line B was obtained using tissue pretreated with labeled hormone.

B) Binding of ^{125}I-hCG to testicular homogenate pretreated either with increasing amounts of unlabeled hCG (0–20 ng) or with 1.25 ng of ^{125}I-hCG. The experimental details for this study were the same as for (A) except that unlabeled hCG was used instead of testicular binding inhibitor. Line A shows the competitive inhibition plot of the binding data obtained using tissue pretreated with unlabeled hCG, and Line B was obtained using tissue pretreated with labeled hormone. All data points represent means of duplicate determinations.

high affinity for ^{125}I-hLH and ^{125}I-hFSH, indicating that they do not bind or compete with the hormones for the gonadotropin binding sites in testicular tissue.

Binding of the inhibitor, or hormone, to the tissue in the first incubation step was not readily exchangeable with that of the subsequent inhibitor, or hormone, added in the second step (Fig. 4A). A similar pattern of binding behavior was observed when the same preincubation experiments were repeated using unlabeled hCG rather than the testicular inhibitor (Fig. 4B); these experiments further support the original evidence of Chen et al. (1979) and a report by Katikineni et al. (1980) demonstrating irreversibility of the hCG and LH binding to particulate gonadal receptors. Thus, our findings strongly suggest that the binding reaction of the testicular inhibitor and LH/hCG binding sites might also be irreversible in nature and might not follow a rapid-equilibrium binding model under in vitro conditions. A conclusive interpretation, however, should wait until the hCG-binding inhibitor is purified and the mechanism of gonadotropin binding reaction is fully elucidated. Interesting in this context is the observation of Kumari et al. (1979) that the ability of a LH/hCG binding inhibitor obtained from extracts of porcine corpus luteum to inhibit progesterone secretion in cultured granulosa cells is not reversed after addition of LH. This would suggest that binding irreversibility may also be an inherent property to other gonadotropin binding inhibitors. This possibility should be closely studied.

It is becoming increasingly clear that gonadotropins can regulate the concentration of their specific binding sites in gonadal tissue, an effect which cannot be explained by receptor-occupancy (Rojas and Bhalla, 1979; Bhalla et al., 1979; Chen et al., 1979; Catt et al., 1979; Huhtaniemi et al., 1979; Sharpe, 1977). Still, little is known about the mechanism whereby such changes in the availability of the receptor are induced. An attempt was made to see whether a relationship exists between the content of the hCG-binding inhibitor in the testis of hCG-treated rats and the concomitant reduc-

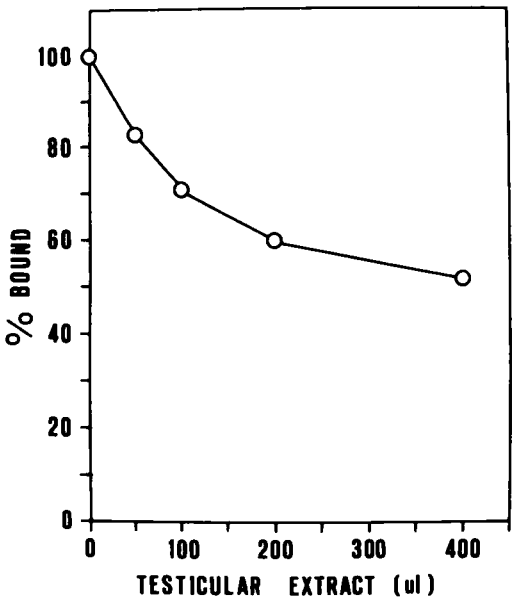

FIG. 5. Effects of the hCG binding inhibitor extracted from rat testis on the specific binding of ^{125}I-hCG to the 2000 X g pellets of ovarian homogenate. ^{125}I-hCG (1.25 ng) was incubated with ovarian homogenate (10 mg, wet tissue weight) in the presence of 0, 25, 100, 200 and 400 μl of testicular extract. The final volume of the reaction mixture was 1 ml in albumin/phosphate buffer. After incubation at 37°C for 2 h, the tubes were processed as described in Materials and Methods. All values represent the mean of duplicate determinations.

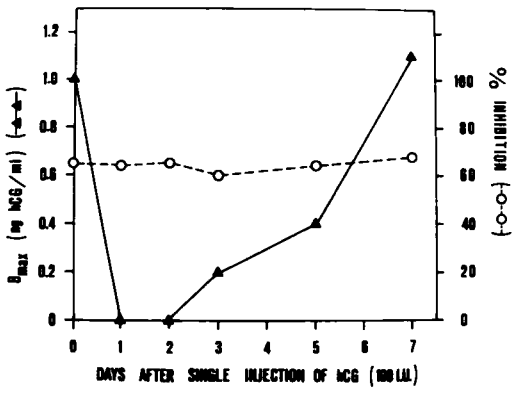

FIG. 6. hCG Binding inhibitory activity and concentration of LH/hCG binding sites in testicular tissue prepared from rats sacrificed at different time intervals following a single injection of hCG (100 IU). For measurement of hCG binding inhibitory activity, 200 μl of pooled tissue extract obtained from testes of four treated rats were incubated with 20 mg of testicular homogenate and 1.25 ng of ^{125}I-hCG in the standard binding assay. Results were expressed as percentage of binding inhibition. B_{max} values plotted were obtained by Scatchard analysis of the data generated from binding assays described under Materials and Methods. The B_{max} value for controls (animals which received injection of saline) is shown in Day 0. Each data point represents triplicate determinations.

tion of LH/hCG receptors (Fig. 6). Results revealed that the down-regulation of receptors proceeds without significant changes in the hCG binding inhibitor content in the testis, suggesting that the two phenomena, i.e., hCG inhibitory activity and gonadotropin-induced reduction in availability of LH/hCG receptors, were not necessarily related to one another. A lack of correlation between the gonadotropin receptor content and the quantity of extractable FSH binding inhibitor has also been reported. O'Shaughnessy (1979) found that the increase in the concentration of the FSH-binding inhibitor observed in maturing rats was not related to the significant decrease in FSH binding receptor sites which also occurs during the same period.

The detection of the hCG binding inhibitory activity in many tissues which do not necessarily bind LH/hCG specifically (Catt et al., 1976; Saxena and Rathnam, 1976) demonstrated a lack of tissue specificity that puts in serious doubt its physiological significance (Table 2). Reichert and Abou-Issa (1977) have reported that buffer extracts of rat liver and kidney inhibit binding of ^{125}I-hFSH to testicular receptors. Yang et al. (1979) have found that direct rat liver extract inhibits LH binding to both ovarian and testicular receptors. The presence of an inhibitor of ovarian extracts from pregnant rats having the ability to interfere with LH/hCG binding to the testicular receptor was not observed by Yang et al. (1976b). These authors reported that ovarian extracts of pregnant rats did not inhibit the LH binding to rat testis, even when up to 40 mg of ovarian equivalent were tested in a binding reaction volume of 0.22 ml (Yang et al., 1976b). In contrast, we were able to demonstrate that 200 mg tissue equivalent of ovaries from pregnant rats in a 1 ml reaction mixture inhibited the hCG binding to testicular receptors by 50% (Table 2). The reason for this discrepancy is not clear. Whether the binding inhibitory substance present in these ovarian extracts is different from the LHRBI described by Yang et al. (1976a,b) has yet to be elucidated.

TABLE 2. Effects of the extracts of various tissues and serum from rat on the specific binding of ^{125}I-hCG to testicular receptors.

Source of extracts[a]	% Inhibition of ^{125}I-hCG binding[b]	
	Direct extract	Heated extract[c]
Ovary	34.7	0
Ovary of pregnant rats	50.3	8.0[d]
Testis	47.6	18.6[e]
Kidney	18.2	0
Liver	21.4	0
Brain	20.0	0
Serum of male rats	44.8	7.1[d]
Serum of castrated male rats	50.1	0
Serum of female rats	37.7	0
Serum of pregnant rats	46.2	0

[a]Tissue extracts and sera pooled from several rats were obtained as described in Materials and Methods. The quantity of the extracts was 200 μl for ovaries and 100 μl for other tissue extracts and serum. The tissue equivalent was 100 mg for the extracts. Two hundred microliters of ovarian extract was used because this extract was prepared by homogenization in a tissue:volume ratio of 1 g/2 ml buffer instead of 1 g tissue/1 ml utilized for the preparation of other extracts (for details, see Materials and Methods).

[b]Reaction mixture containing 1.25 ng ^{125}I-hCG, 200 μl of testicular homogenate, and an aliquot of tissue extract or serum in a final volume of 1 ml with albumin/phosphate buffer. Results are expressed as a percentage inhibition of the ^{125}I-hCG binding in the presence of control buffer alone. Data are the mean of three determinations.

[c]Extracts and serum were heated in a boiling water bath for 30 min.

[d]$P>0.05$ vs control.

[e]$P<0.05$ vs control.

Although the significance and origin of the LH/hCG binding inhibitor found in rat testis remain to be determined, because LH/hCG receptors are essentially localized in gonads, it is tempting to consider that the presence of such an inhibitor in these tissues could be of particular importance for the action of gonadotropins. In any event, its potential use as a tool allowing manipulation of LH/hCG binding to gonadal receptors appears to be self-evident. Work in progress is expected to clarify some of these open questions.

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