Biological Activity of Human Chorionic Gonadotropin
Released from Testis Binding-Sites

(*I*-gonadotropin/receptors/membrane/cyclic AMP/steroidogenesis)

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ABSTRACT The effect of testicular binding of human chorionic gonadotropin upon the biological activities of the hormone was examined by comparison of the binding and activation properties of *I*-labeled gonadotropin before and after binding to rat testis in vitro. Biologically active *I*-gonadotropin taken up by rat testis was dissociated from testis binding-sites at low pH and evaluated for its ability to bind again to testis, adenylate cyclase activation, and stimulation of steroidogenesis during subsequent incubation with fresh testis. Binding to tissue receptor-sites for 4 hr did not impair the biological properties of gonadotropin, though hormone remaining in the incubation medium had reduced affinity for tissue binding-sites during subsequent incubation with rat testes. In comparison to the original preparation, *I*-labeled gonadotropin previously eluted from specific binding-sites of rat testis showed significantly increased binding activity and stimulation of cyclic AMP and testosterone release during further incubation with rat testes in vitro. The enhancement of biological activity of the eluted hormone is attributable to affininity purification of the original hormone preparation by selective uptake at receptor-sites. These results demonstrate that gonadotropin is not inactivated or degraded during combination with gonadotropin receptors of rat testis.

Gonadotropin binding-sites of high affinity for luteinizing hormone and gonadotropin have been demonstrated in subcellular fractions and homogenates of rat testis and ovary (1-3). Such binding is specific, saturable, and temperature-dependent; it is relatively unaffected by physiological variations in calcium concentration of the incubation medium and is associated with membrane fragments during differential centrifugation of testis-binding fractions (4). In the present studies, the binding properties of *I*-labeled gonadotropin have been correlated with the in vitro biological activities of the hormone, expressed as stimulation of cyclic AMP formation and testosterone production by isolated rat testis. Gonadotropin-responsive adenylate cyclase of rat testis showed marked lability in homogenates and membrane-rich fractions, and testosterone production by dispersed interstitial cells of rat testis responded poorly to gonadotropins (5). In contrast, the rate of testosterone formation and release by intact, isolated rat testis was extremely sensitive to gonadotrophic stimulation in vitro and responded to agarose-coupled luteinizing hormone (5, 6), indicating the probable location of gonadotropin receptors upon the cell surface. The sensitivity of isolated rat testes to gonadotropins permits accurate evaluation of the biological activity of *I*-labeled gonadotropin in vitro, and comparison of the activity of the labeled hormone with that of the original preparation. Such labeled hormone can be used for direct measurement of hormone uptake by testis binding-sites during in vitro studies on the actions of gonadotropins on testicular steroidogenesis.

To determine the fate of the hormone at receptor sites, *I*-gonadotropin taken up by testis in vitro was eluted and evaluated for three essential aspects of hormonal activity—binding to testicular receptors, activation of adenylate cyclase, and stimulation of testosterone synthesis.

MATERIALS AND METHODS

Preparation of *I*-Labeled Gonadotropin. Gonadotropin preparations (7,400-10,000 IU/mg) were labeled with *I* by a modification of the chloramine-T procedure (1, 7), with purification by chromatography on a 14 X 0.5-cm column of Agarose-concanavalin A prepared by coupling 500 mg of concanavalin A to 50 g of Sepharose 6B activated with cyanogen bromide (8). After elution of free iodide with 12 ml of phosphate-buffered saline containing 1 mg/ml of bovine gammaglobulin, the labeled hormone was eluted with 0.2 M o-methyl-o-glucopyranoside. When 1-ml fractions were collected, free iodide appeared in fractions 6-8, and a small quantity of "damaged" tracer in fractions 14-18, followed by tracer with maximum binding to antibody and testis binding-sites in fraction 20-26. These fractions were pooled, and tracer mass was determined by solid-phase radioimmunoassay in antibody-coated polystyrene tubes (9). Biological activity of the labeled gonadotropin preparations was evaluated in vitro by radioimmunoassay of testosterone and cyclic AMP released into the incubation medium by isolated rat testis during incubation at 34° for 4 hr in Krebs-Ringer bicarbonate buffer containing 1 mg/ml of glucose (6).

In experiments where cAMP release was measured, incubations were for 2 hr in Krebs-Ringer bicarbonate-glucose buffer containing 5 mM of theophylline. After incubation, 25- to 100-μl aliquots of medium were assayed for cyclic AMP content by a modification of the radioimmunoassay method of Steiner et al. (10); dioxane was used for the separation of bound and free tracer.

Binding Studies. Testes from mature male rats were decapsulated and incubated with labeled gonadotropin in 2 ml of Krebs-Ringer bicarbonate buffer in glass counting vials shaken (at 150 cycles/min) for 4 hr at 34° under 95% O2-5% CO2. The testes were washed twice with cold PO4-buffered NaCl and counted in an automatic gamma spectrometer with a counting efficiency of 50% for *I*. In each experiment, incubations with excess gonadotropin (500 IU) were performed to establish the nonspecific uptake of tracer, which was subtracted from each sample. The binding properties of *I*-gonadotropin remaining in the incubation medium were also examined by further incubation of aliquots of medium with fresh decapsulated testes.
Elution of $^{125}$I-Gonadotropin from Testis Binding-Sites. After incubation with 5 x $10^4$ cpm $^{125}$I-gonadotropin for 4 hr, intact testes were washed twice with phosphate-buffered saline and shaken at 4° in 0.15 M NaCl (pH 2.3, adjusted with HCl) for 16 hr. The bound tracer was not significantly reduced by incubation in buffer alone, nor by subsequent incubation with 100 IU of unlabeled gonadotropin, 1 M EDTA, or 1 M α-methylglucopyranoside. Incubation at pH 2.3 released 40-50% of the bound hormone; the pH of the medium rose to 5.6 at the end of incubation.

RESULTS

Biological activity of $^{125}$I-gonadotropin

Testosterone production during incubation of rat testes with different amounts of $^{125}$I-labeled gonadotropin (200,000 dpm/ng; 90 μCi/μg) and unlabeled gonadotropin (0.25-5 ng) gave parallel dose-response curves (Fig. 1), with potency relative to the original tracer of 1.19 (95% confidence limits, 0.84-1.47). Such biologically active tracer was used for the binding studies shown in Table I. The data shown in Fig. 2 were derived from a different preparation of lower specific activity (36,000 dpm/ng) and equivalent biological activity.

Binding of original and eluted $^{125}$I-gonadotropin

During incubation with fresh testes (Table I), the uptake of tracer eluted from binding-sites was significantly greater than that of the original tracer (Experiment A: 3-fold; $P<0.01$ by Student's t-test. Experiment B: 2.1-fold; $P<0.01$). When $^{125}$I-gonadotropin was eluted from testis binding-sites by two consecutive incubations at pH 2.3, the less readily-eluted tracer showed greater uptake than tracer eluted during the first period of incubation (Fig. 2). Tracer remaining in the incubation medium after uptake studies showed consistently lower uptake than the original tracer when incubated with fresh testes. Residual gonadotropin previously eluted from testis binding-sites was taken up from the medium by fresh testes to a significantly greater extent than residual gonadotropin after incubation with the original tracer (1.7- to 1.8-fold, $P<0.001$).

Biological activity of original and eluted $^{125}$I-gonadotropin

Comparison with the steroidogenic activity of the original labeled hormone revealed complete retention of biological

**TABLE 1. Specific uptake of $^{125}$I-gonadotropin before and after elution from testis binding-sites**

<table>
<thead>
<tr>
<th>$^{125}$I-gonadotropin preparation</th>
<th>% Uptake by testis (mean ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First incubation</td>
<td>Second incubation</td>
</tr>
<tr>
<td>Original gonadotropin</td>
<td>17.5 ± 1.4</td>
</tr>
<tr>
<td>Eluted gonadotropin</td>
<td>51.6 ± 4.5</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
</tr>
<tr>
<td>Original gonadotropin</td>
<td>23.3 ± 3.1</td>
</tr>
<tr>
<td>Eluted gonadotropin</td>
<td>48.6 ± 2.9</td>
</tr>
</tbody>
</table>

In both experiments, the percentage uptake of $^{125}$I-gonadotropin was constant over the range used (50,000-300,000 dpm; 0.25-1.5 ng). The values for mean uptake ±SD were calculated from the results of 16 replicates in each group of testes in Exp. A and 12 replicates in Exp. B, after subtraction of the nonspecific uptake (13.5%) observed in the presence of excess gonadotropin.

![Fig. 1. Testosterone production by rat testis in vitro during incubation for 4 hr with unlabeled gonadotropin and $^{125}$I-labeled gonadotropin. Each point shows the mean ±SD of quadruplicate incubations. O—O, gonadotropin; O—O, $^{125}$I-gonadotropin.](image)

![Fig. 2. Testis binding of $^{125}$I-gonadotropin and stimulation of cyclic AMP and testosterone release during incubation for 2 hr at 37°. The effects of 10⁵ dpm (3 ng) of the original tracer are compared with those of $^{125}$I-gonadotropin recovered from testis binding-sites during two successive elutions at pH 2.3. The nonspecific uptake of $^{125}$I-gonadotropin by control testes was determined by addition of 500 IU of gonadotropin before incubation; control testes for cyclic AMP and testosterone release were incubated without added hormone. Shaded areas, control values; open areas, plus $^{125}$I-gonadotropin.](image)
potency by the eluted ¹²⁵I-gonadotropin (Table 2); in fact, steroidogenic activity was significantly increased in both experiments, by a factor of 1.40 (95% confidence limits 1.04–1.92) in experiment A, and 1.44 (95% confidence limits 1.54–1.85) in experiment B.

When cAMP release by testis was measured by radioimmunoassay, the eluted gonadotropin was again more active than the original hormone, and greater stimulation was observed with the gonadotropin eluted from binding-sites (Fig. 2). This experiment also showed a correlation between gonadotropin binding and cAMP formation, while testosterone production remained at its maximum level; the quantity of gonadotropin used for these studies (3 ng) was at the upper level of the dose-response range of rat testis in vitro to this hormone (5, 6).

**DISCUSSION**

The biological activity of gonadotropin labeled with ¹²⁵I in the presence of low concentrations of chloramine T at 4°C and purified by cellulose chromatography is retained, as measured by in vivo bioassays of the labeled hormone (7). Radioiodinated gonadotropin used in the present studies was also demonstrated to retain full steroidogenic activity upon isolated rat testis, thus providing a valid tracer for simultaneous measurements of hormone binding and biological activities in vitro. In particular, it was possible to compare the properties of the original labeled preparation with those of gonadotropin taken up by testis binding-sites and with unbound hormone remaining in the incubation medium.

The labeled gonadotropin eluted from testis binding-sites at low pH showed no loss of activity, as judged by three criteria that are known to be essential for the action of peptide hormones on their target cells. The eluted hormone displayed increased specific uptake by testis binding-sites and increased ability to stimulate cyclic AMP formation and testosterone synthesis in vitro. The detectable enhancement of these properties of the labeled hormone was attributable to selective uptake of the most-active hormone from the gonadotropin preparations used for labeling. Labeled gonadotropin eluted during a second incubation at pH 2.3 (more tightly bound gonadotropin) showed further enhancement of in vitro binding activity and stimulation of adenylate cyclase, indicating that the hormone eluted from higher-affinity binding-sites was also the most biologically active hormone. Such increased biological activity of gonadotropin eluted from binding-sites is consistent with the original activity of the gonadotropin preparations used for labeling with ¹²⁵I. From the starting activity of 7,400–10,000 IU/mg, the potency of the tracer eluted from testis binding-sites has been increased toward the maximum level of 15,000–18,000 IU/mg observed in freshly prepared batches of highly purified gonadotropin. The correlation between improved binding of the eluted gonadotropin fractions and cyclic AMP formation in the presence of constant maximum testosterone release suggests that only a small proportion of the active receptor-sites in rat testis need be occupied by trophic hormone to produce a full biological response.

Preservation of biological activity during residence on tissue binding-sites has also been observed with insulin after binding to liver- and fat-cell membranes (11, 12), and with glucagon after binding to liver-cell membranes (13). Labeled glucagon remaining in the incubation medium has been reported to be inactivated (13), and medium insulin reported to retain biological activity (12). The present studies have shown that three important biological properties of labeled gonadotropin are preserved and enhanced during binding to tissue receptor-sites, and that hormone remaining in the incubation medium has appreciably reduced binding affinity for testis. The enhanced biological activities of the eluted hormone represents an example of purification by affinity chromatography on tissue receptor-sites.