

Temporal Characteristics of Gonadotropin Interaction with Rabbit Luteal Receptors and Activation of Adenylyl Cyclase: Comparison to the Mode of Action of Catecholamine Receptors*

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ABSTRACT. The temporal characteristics of gonadotropin (LH and hCG) and catecholamine interaction with their luteal receptors and activation of adenylyl cyclase were studied in rabbit luteal membranes. Studies on hormone-receptor interaction showed that, once bound, [¹²⁵I]iodo-hCG dissociated from its receptor very slowly. If excess LH was added 30 min after the initiation of [¹²⁵I]iodo-hCG binding, 85% of the [¹²⁵I]iodo-hCG bound at 30 min was still bound to the luteal receptors 4.5 h later. The rate of dissociation of [¹²⁵I]iodo-hCG from its receptor was not altered by 100 μM GTP, 2 mM MgCl₂, or GTP plus MgCl₂. The slow rate of [¹²⁵I]iodo-hCG dissociation observed at 30 min was not due to a time-dependent change in the hormone-receptor complex, as the dissociation of [¹²⁵I]iodo-hCG was equally slow 5 min after the initiation of the binding reaction. Studies on the activation of luteal adenylyl cyclase by LH

showed that stimulation by 1 μg/ml ovine LH (oLH) could be prevented but, once initiated, could not be reversed by antiserum to oLH. This indicates that once bound to the rabbit luteal LH receptor, oLH causes persistent activation of rabbit luteal adenylyl cyclase. In contrast, the activation of luteal adenylyl cyclase by 1 μM isoproterenol could be completely reversed by the addition of 50 nM propranolol 5 min after the initiation of the reaction. The inhibitory effect of the propranolol could be completely overcome by the addition of excess isoproterenol, indicating that catecholamine binding to its luteal β-receptor is readily reversible. Thus, there appears to be a basic difference in the mechanism by which the gonadotropins and catecholamines interact with their receptors and activate the rabbit luteal adenylyl cyclase. (*Endocrinology* 111: 970, 1982)

IT IS now well established that the initiation of LH/hCG action in the ovary is dependent upon the binding of hormone to specific receptors located on the surface of ovarian cells. The first detectable biological response upon hormone occupancy of these gonadotropin receptors is an increase in intracellular cAMP levels due to activation of adenylyl cyclase, followed by an increased steroidogenic output (1, 2). However, conflicting reports have appeared in the literature with regard to the reversibility of the interaction of gonadotropin with its receptor. Studies in the cow indicate that [¹²⁵I]iodo-ovine LH ([¹²⁵I]iodo-oLH) (3) and [¹²⁵I]iodo-hCG (4) readily dissociate from luteal receptors. In contrast, neither [¹²⁵I]iodo-hLH (5) nor [¹²⁵I]iodo-hCG (6, 7) was found to dissociate readily from rat ovarian receptors. Similarly, a disparity is observed with regard to gonadotropin stimulation of steroidogenesis. While in normal rats (8) and

mice (9), stimulation by oLH, but not hCG, of Leydig cell steroidogenesis is readily reversed upon washing the cells, it is not clear whether stimulation of steroidogenesis by oLH in murine Leydig tumor cells is reversible. Moyle and coworkers (10, 11) reported a lack of reversal upon washing of cells and that the addition of antisera to oLH results in termination of the steroidogenic response. In contrast, Segaloff *et al.* (9) reported that mouse Leydig tumor cells respond like normal mouse Leydig cells in that the stimulation of steroidogenesis by oLH, but not hCG, is readily reversed upon washing the cells free of gonadotropin. Although the actions of LH and hCG are mediated via the stimulation of adenylyl cyclase, the reversibility of the stimulation of this enzyme system by gonadotropin has not been investigated. In those systems that have been examined, such as the glucagon-stimulated rat liver adenylyl cyclase (12) and the catecholamine-stimulated kitten heart adenylyl cyclase (13), hormonal stimulation of adenylyl cyclase is readily reversible. Since the rabbit corpus luteum has an adenylyl cyclase system that is responsive to both LH and catecholamines (14) and both of these hormones activate the same adenylyl cyclase (15), we compared the interaction

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of the gonadotropins and catecholamines with their receptors and examined whether hormonal activation of rabbit luteal adenylyl cyclase was reversible. A preliminary account of some of these results has been presented (16).

Materials and Methods

Materials

Inorganic ^{32}P was purchased from Union Carbide (Tuxedo, NY), ^{125}I (carrier-free) was obtained from Iso-Tex Diagnostics (Friendswood, TX), and $[^3\text{H}]\text{cAMP}$ was purchased from Schwarz/Mann (Orangeburg, NY). ATP (Na-salt; catalog no. A-2383), GTP, cAMP, EDTA, Tris, creatine phosphate, and myokinase (2000 U/mg) were obtained from Sigma Chemical Co. (St. Louis, MO). Creatine phosphokinase (200 U/mg) was purchased from Calbiochem (LaJolla, CA). Highly purified hCG (hCG-CR119) and oLH (NIH-LH-S19) were obtained from the NIH. (-)Isoproterenol was a gift from Dr. Nachod of Sterling-Wintrop Laboratories (Rensselaer, NY). (-)Propranolol and the hCG used to induce pseudopregnancy were gifts from Ayerst Laboratories (New York, NY). All other chemicals and reagents were of the highest commercially available purity and were used without further purification.

$[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (SA, >50 Ci/mmol) was synthesized according to the procedure of Walseth and Johnson (17) and was purified by DEAE-Sephadex A-25 chromatography as described previously (18). $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ prepared by this procedure was supplied by the Core Laboratory on Cyclic Nucleotide Research, Center for Population Research and Studies on Reproductive Biology, Baylor College of Medicine (Houston, TX).

$[^{125}\text{I}]\text{Iodo-hCG}$ was prepared using the lactoperoxidase procedure, as previously described (19). $[^{125}\text{I}]\text{Iodo-hCG}$ prepared by this method had between 0.82–0.95 mol ^{125}I /mol hCG (SA, 40–46.5 $\mu\text{Ci}/\mu\text{g}$) and showed a biological potency in adenylyl cyclase assays that was indistinguishable from that of uniodinated hCG. Bindability of the labeled hormone in the presence of excess receptor-containing membranes was determined on each batch and was found to vary between 60–65% (19).

Antiserum to oLH (lot KPY-1-27-D) was prepared in rabbits. The immunoglobulin G fraction of the antiserum was partially purified by precipitation with ammonium sulfate at 40% saturation, followed by chromatography over DEAE-cellulose and two sequential gel permeation chromatographies over Sephadex G-200. The final fraction was concentrated by precipitation with ammonium sulfate (50% saturation) and dialyzed against 10 mM PBS, pH 7.3. The partially purified immunoglobulin G fraction thus obtained (28 mg protein/ml) was aliquoted and stored at -70 C until used. Throughout this manuscript, this fraction is referred to as anti-LH serum or simply anti-LH. Anti-LH prepared by this method was provided by Dr. Darrell N. Ward, Department of Biochemistry, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute (Houston, TX).

Animals

New Zealand White rabbits (3.0–4.5 kg) were used throughout. Pseudopregnancy was induced by injecting 100 IU hCG

(Ayerst) in saline, iv. The rabbits were killed by cervical dislocation on day 7 of pseudopregnancy (the day of hCG injection was day 0). The ovaries were removed and placed in ice-cold Krebs-Ringer bicarbonate, pH 7.4, until dissection of the corpora lutea. The dissected corpora lutea were homogenized, and membrane particles were prepared as previously described (14).

Adenylyl cyclase assays

Adenylyl cyclase activity was determined at 32.5 C in medium containing the indicated concentration of ATP (with 10^7 cpm $[\alpha\text{-}^{32}\text{P}]\text{ATP}$), 5 mM MgCl_2 , 1.0 mM EDTA, 1.0 mM cAMP (with approximately 10,000 cpm $[^3\text{H}]\text{cAMP}$), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, and 25 mM Tris-HCl, pH 7.5. Fixed time assays were performed in a final volume of 50 μl and were carried out for 10 min. These assays were stopped by the addition of 100 μl stopping solution, consisting of 10 mM cAMP, 40 mM ATP, and 1% sodium dodecyl sulfate. Time courses of cAMP accumulation were carried out by incubating larger volumes, removing 50- μl aliquots at the indicated times, and stopping the reaction by addition to 100 μl of the above-described stopping solution. The $[^{32}\text{P}]\text{cAMP}$ formed and the $[^3\text{H}]\text{cAMP}$ added to monitor recovery were isolated, according to the method of Salomon *et al.* (20), using Dowex and alumina chromatography, as modified by Bockaert *et al.* (21).

$[^{125}\text{I}]\text{Iodo-hCG}$ binding assay

$[^{125}\text{I}]\text{Iodo-hCG}$ binding assays were carried out in the presence of 65–80 pM $[^{125}\text{I}]\text{iodo-hCG}$, 1.25% BSA, 1.0 mM EDTA, and 25 mM Tris-HCl, pH 7.5, in a volume of 2.0 ml at 32.5 C. When present, the concentration of MgCl_2 was 2 mM and that of GTP was 100 μM . At the indicated time intervals, 50- μl aliquots were withdrawn and added to 1.5 ml ice-cold 2 mg/ml bovine γ -globulin in 0.1 M NaCl, followed by the addition of 0.5 ml ice-cold 20% polyethylene glycol (Carbowax 6000). The mixtures were centrifuged for 10 min in the cold at 3000 rpm in a Sorvall RC-3 centrifuge (Ivan Sorvall, Inc., Norwalk, CT) affixed with an HL-8 rotor, and the supernatants were discarded by aspiration. The precipitates were resuspended by the addition of 1.5 ml 0.1 M NaCl and were reprecipitated by the further addition of 0.5 ml 20% polyethylene glycol. The amount of $[^{125}\text{I}]\text{iodo-hCG}$ bound to the precipitates was determined after centrifugation and aspiration of the supernatant fluids. Values of $[^{125}\text{I}]\text{iodo-hCG}$ bound presented in the time courses correspond to the total amount bound without subtraction of reaction blanks of any kind.

Protein was determined by the method of Lowry *et al.* (22) using crystalline BSA (fraction V) as standard.

All data in the text and figures are from representative experiments; each experiment was repeated at least three times with similar results.

Results

Temporal characteristics of $[^{125}\text{I}]\text{iodo-hCG}$ binding

To determine whether $[^{125}\text{I}]\text{iodo-hCG}$ forms a reversible complex with its receptor, we examined the time

course of [125 I]iodo-hCG binding to and possible dissociation from rabbit luteal receptors. Dissociation studies were initiated after either 30 or 5 min of incubation by the addition of excess unlabeled oLH (20 μ g/ml) alone or in combination with 2 mM MgCl₂, 100 μ M GTP, or 2 mM MgCl₂ plus 100 μ M GTP. Changes in the amount of [125 I]iodo-hCG bound to the luteal membranes were monitored throughout the next 270 or 95 min.

As seen in Fig. 1A, the binding of [125 I]iodo-hCG to

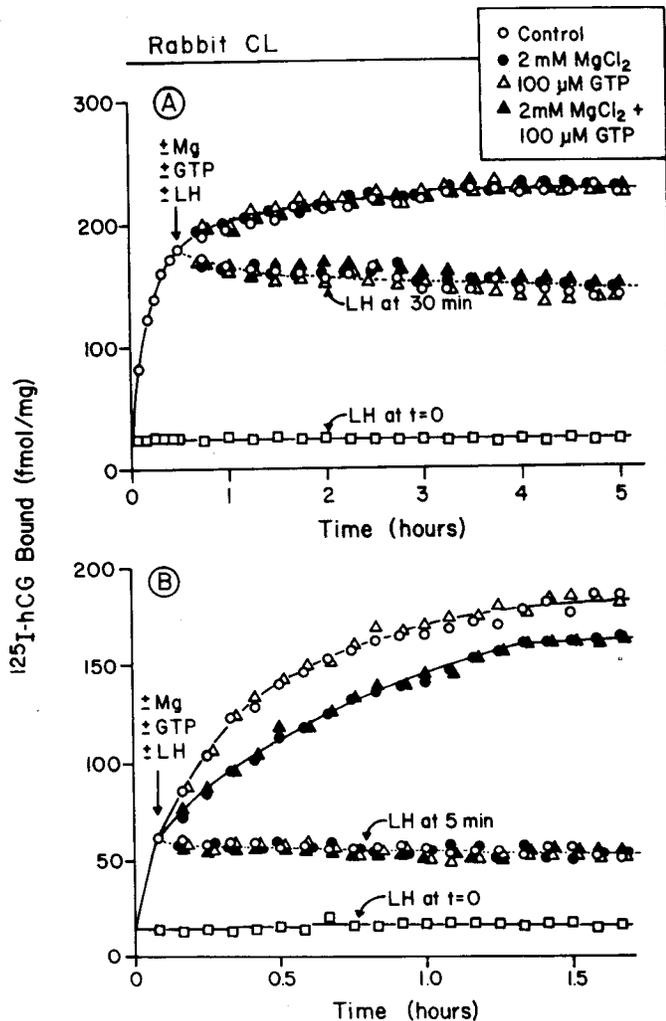


FIG. 1. Time course of [125 I]iodo-hCG interaction with rabbit luteal gonadotropin receptors. A, Rabbit luteal membranes were incubated with 79 pM [125 I]iodo-hCG as indicated in *Materials and Methods*. At 30 min, 2 mM MgCl₂, 100 μ M GTP, 2 mM MgCl₂ plus 100 μ M GTP with and without 20 μ g/ml oLH were added as indicated. To one curve (\square), 20 μ g/ml oLH were added at zero time. The membrane protein concentration was 0.25 mg/ml. B, Rabbit luteal membranes were incubated with 65 pM [125 I]iodo-hCG as described in *Materials and Methods*. The rest of the conditions are the same as those described in A, except that the later additions to the assay were made at 5 min, and the membrane protein concentration was 0.21 mg/ml. CL, Corpus luteum. The data in this and other figures are from representative experiments; each experiment was repeated with similar results at least three times.

luteal receptors increased in a time-dependent manner in the absence of excess oLH. The addition of Mg, GTP, or combinations thereof at 30 min did not alter the further binding of [125 I]iodo-hCG. When excess oLH was added at 30 min, the dissociation of bound [125 I]iodo-hCG from its receptor was very slow. After 4.5 h of incubation in the presence of excess oLH, 85% of the [125 I]iodo-hCG bound at 30 min was still bound to the luteal receptors. This concentration of oLH when added at zero time completely prevented the [125 I]iodo-hCG from binding to the luteal gonadotropin receptors. In other studies in which the dissociation was followed for 24 h, there was no further decrease in the amount of [125 I]iodo-hCG bound after 4.5 h of incubation (not shown). The addition of Mg, GTP, or combinations thereof together with excess oLH did not alter the rate of dissociation of [125 I]iodo-hCG from its receptor (Fig. 1A).

We next determined whether the tight binding observed with [125 I]iodo-hCG to luteal gonadotropin receptors might be due to a slow time-dependent change in the nature of the hormone-receptor complex occurring during the 30-min incubation, such as has been observed by Ross *et al.* (23) for the binding of the β -adrenergic blocker iodohydroxybenzylpindolol. Figure 1B shows that even if the dissociation study was initiated after only 5 min of incubation, the bound label did not dissociate readily regardless of the addition of GTP, Mg, or a combination thereof. In agreement with our previous findings that Mg slightly lowers the affinity of hCG for the luteal receptor and GTP has no effect on hCG binding (19), the addition of GTP (100 μ M) at 5 min in the absence of oLH had no effect on the subsequent binding of [125 I]iodo-hCG, and the addition of either Mg (2 mM) or GTP plus Mg (100 μ M and 2 mM, respectively) lowered the occupancy of receptor by [125 I]iodo-hCG.

Persistent activation of adenylyl cyclase by oLH

The finding that [125 I]iodo-hCG appears to interact in an essentially irreversible manner with its receptor was reminiscent of the findings of Garfink *et al.* (8) and Segaloff *et al.* (9). In these studies, stimulation of steroidogenesis in normal Leydig cells by hCG was not readily reversed upon washing the cells. However, these authors also observed that stimulation of steroidogenesis by oLH was readily reversed if oLH was used instead of hCG. The following study was performed to determine 1) whether the properties of gonadotropin binding to the corpus luteum are similar to the properties of gonadotropin stimulation of steroidogenesis in normal Leydig cells, 2) whether the tight binding we observed in the corpus luteum was due to the [125 I]iodo-hCG used as a receptor probe, and 3) whether tight binding was a property of the luteal gonadotropin receptor.

To explore the reversibility of oLH stimulation of adenylyl cyclase, we made use of antiserum directed against oLH. The anti-LH used was first titrated against oLH using two different procedures. In the first procedure, we determined the appropriate dilution of antiserum that neutralizes the ability of 1 $\mu\text{g}/\text{ml}$ oLH to stimulate adenylyl cyclase activity. It was found that this was obtained at a 1:20 dilution of the stock antiserum solution (see *Materials and Methods*) in the adenylyl cyclase assays (not shown). The second procedure was to determine the effect of a 1:20 dilution of anti-LH on the ability of varying concentrations of oLH to stimulate adenylyl cyclase activity. It was found that this dilution of antibody was capable of neutralizing the stimulatory activity of 3.12 $\mu\text{g}/\text{ml}$ oLH (not shown).

Figure 2 depicts the results of an experiment in which we attempted to neutralize the stimulatory effects of 1 $\mu\text{g}/\text{ml}$ oLH by the later addition of a 1:10 dilution of the anti-LH after initiating the reaction. Such an addition failed to reverse the stimulatory effect of the hormone over the next 15 min (Fig. 2, first pair of curves). This was not due to an inability of the antiserum to neutralize the LH, since the addition of 1 $\mu\text{g}/\text{ml}$ oLH 5 min after the addition of the antiserum did not result in stimulation of adenylyl cyclase activity even though this concentration of LH could stimulate the enzyme when added after 10 min of incubation (Fig. 2, third pair of curves). Further the antibody itself did not alter the LH receptor, since excess oLH overcame its effect (Fig. 2, second pair of curves). Finally, the antiserum did not alter the basal activity of the enzyme (Fig. 2, fourth pair of curves). In other studies in which the attempted reversal was followed for 30 min, antiserum to oLH failed to reverse the stimulatory effects of 1 $\mu\text{g}/\text{ml}$ oLH (not shown). Thus, it would appear that LH irreversibly activates rabbit luteal adenylyl cyclase.

Reversible activation of adenylyl cyclase activity by catecholamines

To determine whether other luteal receptors linked to the same adenylyl cyclase also caused persistent activation of adenylyl cyclase, we examined the reversibility of isoproterenol stimulation of rabbit luteal adenylyl cyclase. The stimulatory effect of 1 μM isoproterenol on adenylyl cyclase activity could be completely reversed by the addition of 50 nM propranolol 5 min after the initiation of the reaction (Fig. 3). Further, the addition of 100 μM isoproterenol 10 min after propranolol administration restimulated the enzyme to the same degree as if 100 μM isoproterenol was added to an unstimulated enzyme preparation that had been incubated for 15 min (Fig. 3). Therefore, catecholamine receptors linked to the rabbit luteal adenylyl cyclase form a readily reversible hor-

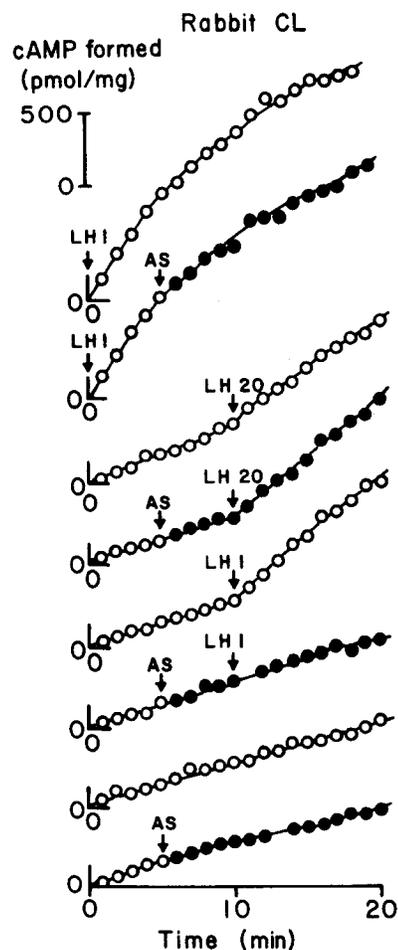


FIG. 2. Lack of effect of LH antiserum on LH-stimulated adenylyl cyclase activity in rabbit corpus luteum (CL) membranes within 15 min of anti-LH addition. Time courses of cAMP accumulation were determined in assay media containing 2.59 mM ATP, 10 μM GTP, 5 mM MgCl_2 , 1.0 mM EDTA, nucleoside triphosphate-regenerating system, oLH, and antiserum-derived LH antibody (AS) as shown. oLH was added at either 1 $\mu\text{g}/\text{ml}$ (LH 1) or 20 $\mu\text{g}/\text{ml}$ (LH 20). The membrane protein concentration was 0.20 mg/ml. Antibody was added to give a final dilution of 1:10 in the assay. ○, Accumulation of cAMP in the absence of AS; ●, accumulation of cAMP after AS addition. From top to bottom: The first pair of curves show that the later addition of AS at 5 min did not result in reversal of the effect of LH 1 (added at zero time). The second pair of curves shows that the later addition of LH 20 at 10 min elicited stimulation of adenylyl cyclase activity regardless of whether AS had been added at 5 min and indicates that antiserum does not interfere with the initiation of the stimulatory event by LH. The third pair of curves shows that AS addition at 5 min blocked the stimulatory action of LH 1 added at 10 min and demonstrates that sufficient antibody was added to block any effect of the free low concentrations of LH used in the first sets of curves. The fourth pair of curves shows that AS addition did not affect the basal activity of the luteal adenylyl cyclase system.

mone-receptor complex, and catecholamine stimulation of adenylyl cyclase in the corpus luteum is as readily reversible as that shown for other β -adrenergic receptor-stimulated systems (13).

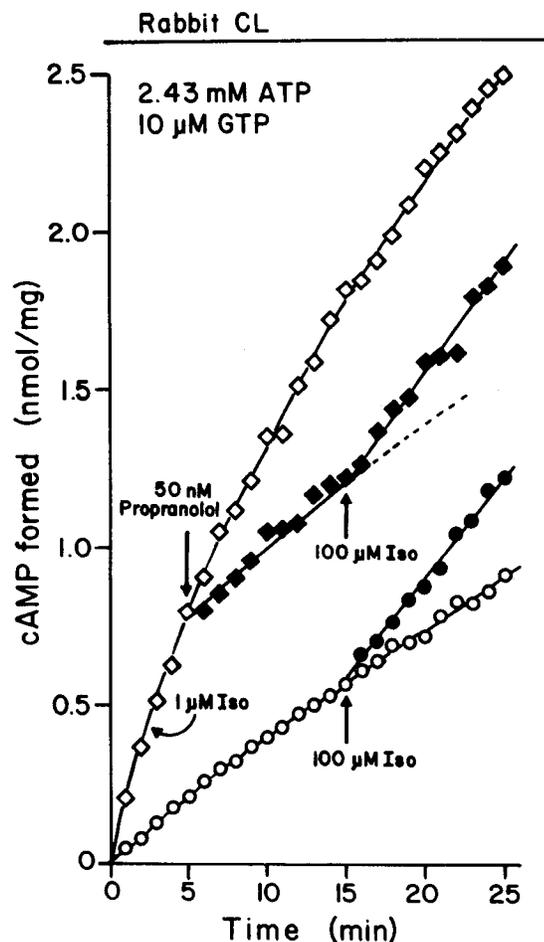


FIG. 3. Reversibility of the catecholamine-mediated stimulation of the rabbit luteal adenylyl cyclase. Time courses of cAMP accumulation were determined, as described in *Materials and Methods*, in the presence of 2.43 mM ATP and 10 μ M GTP. The membrane protein concentration was 0.21 mg/ml. \circ , Incubation carried out without further addition; \diamond , incubation carried out in the presence of 1 μ M isoproterenol (Iso) throughout; \blacklozenge , cAMP accumulation in an incubation that received 1 μ M isoproterenol at zero time, 0.05 μ M propranolol at 5 min, and 100 μ M isoproterenol at 15 min (only the accumulation of cAMP after 5 min is shown, even though the incubation was sampled continuously from zero time on); \bullet , cAMP accumulation in an incubation that received 100 μ M isoproterenol at 15 min. As described above, only the accumulation of cAMP after 15 min is shown, even though the incubation was sampled continuously at 1-min intervals. CL, Corpus luteum.

Discussion

In the present study we have shown that both LH and hCG interactions with the rabbit luteal gonadotropin receptor result in a hormone-receptor complex that is not readily dissociable. Further, the stimulatory action of oLH upon luteal adenylyl cyclase activity cannot be reversed by antiserum directed against oLH when the antiserum is added 5 min after the oLH. In contrast, catecholamine interaction with its receptor and activation of this same luteal adenylyl cyclase are readily

reversible. The inability of anti-oLH to reverse the stimulatory action of oLH upon adenylyl cyclase is most likely due to the failure of oLH to dissociate from its receptor. Under the experimental conditions used in the present study, sufficient antiserum was present to completely neutralize the 1 μ g/ml oLH in the adenylyl cyclase assay. If the oLH had dissociated from its receptor, the antiserum should have bound the oLH, prevented re-binding of the oLH to its receptor, and prevented the further stimulation of adenylyl cyclase activity. The failure of the antiserum to reverse the stimulatory action of oLH was not due to a direct interaction of the antiserum with the LH receptor, since the addition of excess oLH to an adenylyl cyclase assay which contained anti-oLH did not prevent the stimulatory action of the excess oLH. Thus, it would appear that in order to obtain reversal of the stimulatory action of a hormone, it is necessary that the hormone dissociate from its receptor. In agreement with this contention are the studies of Amir-Zaltsman and Salomon (7). These investigators showed that dissociation of bound [125 I]iodo-hCG from rat follicular membranes by acidification results in a reversal of hCG stimulation of adenylyl cyclase activity and that neutralization of the medium resulted in re-binding of the [125 I]iodo-hCG and restimulation of adenylyl cyclase activity. In addition, Strickland *et al.* (24) found that the differential effects of hCG and oLH on the persistent activation of steroidogenesis are correlated with the degree of binding reversibility.

Tight binding of gonadotropin to ovarian receptors is not unique to the rabbit corpus luteum. It also has been demonstrated for rat luteal (5, 6) and follicular (7) gonadotropin receptors. The rate of dissociation of [125 I]iodo-hCG from rabbit and rat (6, 25) ovarian gonadotropin receptors is not increased by added guanine nucleotide. Therefore, one might suggest that tight binding is due to a lack of guanine nucleotide sensitivity of hormone-receptor interactions, since rabbit luteal β -adrenergic receptors are regulated by guanine nucleotides (19) and one role of guanine nucleotide is to increase the rate of dissociation of hormone from its receptor (26). However, this does not appear to be the case. Gonadotropin binding to bovine luteal membranes is not regulated by guanine nucleotides (4), and both [125 I]iodo-hCG (4) and [125 I]iodo-oLH (3) readily dissociate from bovine luteal gonadotropin receptors. Therefore, another mechanism must be responsible for tight gonadotropin binding.

The results of the present study indicate that the lack of [125 I]iodo-hCG dissociation does not develop in a slow time-dependent manner, since tight binding is observed after 5 min as well as after 30 min of incubation. This is in contrast to the findings of Katikineni *et al.* (27), who showed a time-dependent decrease in the reversibility of

[¹²⁵I]iodo-hCG binding to rat testes. Further, the tightness of [¹²⁵I]iodo-hCG binding to rabbit luteal receptors is not dependent on occupancy of a majority of these receptors, as these membranes contain approximately 450–500 fmol binding sites/mg protein (19), and after 5 min of incubation only 10% of these specific binding sites are occupied.

The principal difference between catecholamine- and gonadotropin-mediated activation of adenylyl cyclase is that catecholamine activation is readily reversed upon dissociation from its receptor, whereas gonadotropin activation of adenylyl cyclase activity is not. In light of this persistent activation that occurs in the presence of LH, desensitization to LH (28–32), receptor down-regulation (32, 33), and internalization of hormone-receptor complexes (34) acquire new significance. These mechanisms may be the sole physiological manner of reversing the LH-activated state. This is not to say that inactivation of the activated state is the sole function of adenylyl cyclase desensitization, receptor down-regulation, and internalization, since desensitization is seen with both the irreversibly stimulating gonadotropins and the reversibly activating catecholamines (35, 36). The mechanism by which desensitization arises is not known. Desensitization often precedes receptor down-regulation, thus indicating that receptor down-regulation is not the cause of desensitization (37, 38). In addition, recent studies by Iyengar *et al.* (38) have shown that desensitization to catecholamines is not associated with a change in the guanine nucleotide-binding regulatory component of adenylyl cyclase. By studying and comparing desensitization to both catecholamines and gonadotropins in the rabbit corpus luteum, the mechanisms responsible for the inactivation of the LH-stimulated state of adenylyl cyclase may be established.

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