Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 5837–5841, October 1980
Biochemistry

Hormone-induced guanyl nucleotide binding and activation of adenylate cyclase in the Leydig cell

(hormone action/testicular interstitial cell/nucleotide regulatory protein/receptor–enzyme coupling)

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Communicated by Roy Hertz, July 14, 1980

ABSTRACT **The adenylate cyclase activity of Leydig cell homogenates and membrane fractions is highly dependent on guanyl nucleotides, and enzyme responses to luteinizing hormone or human chorionic gonadotropin are small in the absence of guanyl nucleotides. However, in the presence of 10 μM guanosine 5′-[β,γ-imido]triphosphate Gpp[NH]p, both hormones consistently stimulated testicular adenylate cyclase activity by up to 200%. Leydig cell membranes bound [^{3}H]Gpp[NH]p at 30°C with high affinity ($K_a = 1.5 \times 10^7$ M^{-1}) and binding capacity of 60 pmol/mg of protein. During kinetic studies, the association rate constant was 1.7×10^6 M^{-1} min^{-1}, and the dissociation constant was 0.038 min^{-1}. In the presence of gonadotropin (10 pM to 10 nM), concentration-dependent increases of 40% to 100% in Gpp[NH]p binding were observed in Leydig cell membranes. Kinetic studies showed that gonadotropin decreased the association rate constant to 0.73×10^6 M^{-1} min^{-1} and the dissociation rate constant to 0.017 min^{-1}, with no effect on the equilibrium binding constant. Thus, the increase in Gpp[NH]p binding was not due to a change in receptor affinity but was attributable to increased availability of nucleotide binding sites. The 50% effective dose for adenylate cyclase activation by gonadotropin in the presence of Gpp[NH]p was identical with that observed for gonadotropin-induced binding of the GTP analog (50 nM). Gonadotropin-induced binding of Gpp[NH]p in Leydig cell membranes may represent interaction with the guanyl nucleotide regulatory site during hormonal activation of adenylate cyclase.**

The mechanism by which peptide hormones stimulate adenylate cyclase is now recognized to involve an essential GTP-dependent step (1). Since the original demonstration of the importance of guanyl nucleotides in hormone action, numerous tissues have been shown to be dependent on GTP for full expression of hormone-stimulated adenylate cyclase. The mechanism by which guanyl nucleotides influence adenylate cyclase enzyme is complex and includes interaction of the catalytic enzyme unit with a nucleotide-binding protein (2–8). The molecular interactions induced by agonist occupancy of β-adrenergic receptors lead to the formation of a receptor–guanyl nucleotide regulatory complex, indicating that both physical and functional coupling are required for catecholamine stimulation of adenylate cyclase activity (9). However, a direct demonstration of the hormone-stimulated nucleotide binding process has not been reported.

The degree of dependence of adenylate cyclase on guanyl nucleotides has not been constant in various tissues, probably reflecting the availability or hydrolysis of GTP in the membrane preparations used for assay of the enzyme. In the Leydig cells of the testis, there is a marked disparity between the large cyclic AMP response of intact cells to luteinizing hormone (LH) or human chorionic gonadotropin (hCG) (10, 11) and the relatively poor response of adenylate cyclase in Leydig cell membranes (12). Also, there has been no published description of LH-induced activation of adenylate cyclase in rat Leydig cell membranes, although effects of LH on the ovarian enzyme have been readily demonstrable (13).

In this report, we describe the guanyl nucleotide dependence of adenylate cyclase in Leydig cell fractions and demonstrate that enhancement of guanyl nucleotide binding occurs during stimulation of the testicular enzyme by gonadotropins.

MATERIALS AND METHODS

Materials. [α-^{32}P]ATP, adenosine 5′-[β,γ-imido]triphosphate App[NH]p and guanosine 5′-[β',γ-imido]triphosphate (Gpp[NH]p) were obtained from International Chemical and Nuclear. ATP and GTP were purchased from Sigma; the ATP preparation was a purified synthetic product known to be low in contaminating GTP (14). Ovine LH and other pituitary hormones were obtained from the National Pituitary Agency, and purified hCG (prepared by R. E. Canfield) was provided by the Contraceptive Development Branch (Center for Population Research, National Institute of Child Health and Human Development).

Interstitial Cell Membranes. Testes from adult male Sprague–Dawley rats were decapsulated and the membrane-rich fragmented interstitial cell fraction was prepared as described (15). This material was used for initial studies on enzyme activation and for the preparation of a purified membrane fraction as described by Mintz *et al.* (16). This procedure, originally used for the isolation of ovarian plasma membranes, gave a mean 8-fold purification [from 89 ± 12 (mean ± SD) to 695 ± 80 fmol/mg of protein] and a 25% yield of Leydig cell membranes from interstitial cell particles. The partially purified membranes were stored in liquid nitrogen and showed no change in nucleotide binding properties or adenylate cyclase activity for up to 6 weeks. The membranes used in these studies were analyzed 1–3 weeks after preparation.

Adenylate Cyclase Assay. Enzyme assays were performed in a final volume of 100 μl; reactions were initiated by the addition of 20 μg of membrane protein previously incubated at 30°C for 10 min. The reaction mixture contained 20 mM phosphocreatine, 100 units of creatine kinase per ml, 5 mM magnesium chloride, 1 mM cyclic AMP, 2 mM ATP, and 2–4 $\times 10^6$ dpm of [α-^{32}P]ATP. After incubation for 15–45 min at 30°C, the reaction was stopped by placing the assay tubes in an ice bath and adding 200 μl of 1 M perchloric acid and 50 μl of 5 mM ATP/5 mM cAMP containing 15,000 cpm of cyclic [^{3}H]AMP. Cyclic AMP was isolated from the cyclase assay mixture by chromatography on Dowex 50 and alumina as described by Salomon *et al.* (17).

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Abbreviations: hCG, human chorionic gonadotropin; LH, luteinizing hormone; App[NH]p, adenosine 5′-[β,γ-imido]triphosphate; Gpp[NH]p, guanosine 5′-[β,γ-imido]triphosphate.

Gpp[NH]p Binding Studies. Binding of [^{3}H]Gpp[NH]p to interstitial membrane fractions was analyzed essentially as described for liver membranes (18). Binding studies were performed at 30°C in a final volume of 200 μl; each assay tube contained Leydig cell membranes (20 μg of protein) suspended in 50 mM Tris·HCl, pH 7.4/0.2 mM App[NH]p containing 10^5 dpm of [*guanylyl*-8-^{3}H]Gpp[NH]p (25 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels). During displacement studies, unlabeled Gpp[NH]p was added over the concentration range of 1 nM to 10 μM, and 100 μM nucleotide was used to determine nonspecific binding; this was always less than 0.1% of the bound radioactivity. Gpp(NH)p bound to the membrane fraction was separated from unbound nucleotide by rapid filtration through 0.45-μm cellulose Millipore filters (HAWP). This was performed by adding 3 ml of ice-cold Dulbecco's phosphate-buffered saline (pH 7.4) to each assay tube and transferring the contents immediately to the filters. The filters were washed twice with 3 ml of ice-cold phosphate-buffered saline and transferred to glass counting vials. After addition of 10 ml of Aquasol (New England Nuclear), the bound tritiated nucleotide was assayed in a liquid scintillation β-spectrometer. Equilibrium binding data were analyzed by an equation relating to the concentration of bound ligand to the total Gpp[NH]p concentration, and kinetic data were analyzed according to the second-order rate equation, by using a nonlinear curve-fitting computer program (19).

LH Receptor Assay. The concentration of LH receptors in Leydig cell membranes was measured with ^{125}I-labeled hCG as described (15), to determine receptor binding capacity, to assess membrane purification, and to compare the numbers of LH receptor sites and nucleotide binding sites.

RESULTS

Effects of Guanyl Nucleotides on Hormonal Activation of Adenylate Cyclase. In previous studies (12), stimulation of rat interstitial cell adenylate cyclase by LH and hCG was shown to be small and inconstant, and the testicular membranes were found to degrade rapidly the ATP substrate present in the assay (unpublished data). The latter effect occurred despite the presence of an ATP-regenerating system and could be inhibited by nonionic detergents (12) or preincubation of the membranes for 10 min. To permit the analysis of hormonal effects on adenylate cyclase, the present studies were performed in partially purified and preincubated testis membranes in which the enzyme activity remained linear for up to 45 min at 30°C.

The rate of cyclic AMP formation in testicular membranes during LH stimulation was markedly influenced by the presence of GTP and Gpp[NH]p (Fig. 1). In the absence of nucleotide, the hormone response was only slightly greater than the basal activity. In contrast, enzyme activation in the presence of fluoride was marked and remained linear for about 45 min. Addition of 10 μM GTP caused only a small increase in basal activity and enhanced the hormone-stimulated activity, both of which were linear for about 45 min. Addition of 10 μM Gpp[NH]p markedly enhanced the rate and extent of the basal and hormone-stimulated activities, increasing the latter to slightly above the fluoride-stimulated response, with linearity of enzyme activities for up to 45 min.

The effects of increasing concentrations of Gpp[NH]p and GTP on basal and fluoride- and hormone-stimulated adenylate cyclase activity are shown in Fig. 2. Concentration-dependent changes in basal activity were observed with both nucleotides, and hormonal stimulation of adenylate cyclase was greatly increased in the presence of Gpp[NH]p. This effect was detectable at 10 nM Gpp[NH]p and reached the maximal fluoride-stimulated level at 10 μM Gpp[NH]p (Fig. 2*A*). Fluoride-stimulated activity was not significantly affected by either of the guanyl nucleotides. The effect of GTP upon basal and LH-stimulated activities showed an initial increase followed by a significant decline at higher concentrations (Fig. 2*B*). The maximal enhancement of adenylate cyclase activity by GTP was much less than that elicited by Gpp[NH]p, and no decrease in activity was seen at higher concentrations of the analog.

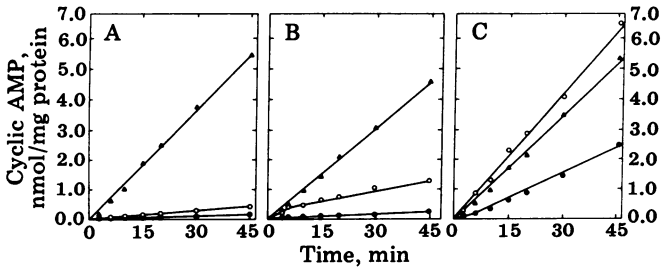

FIG. 1. Time course of adenylate cyclase activation in testis interstitial membranes. Basal (●), LH-stimulated (○), and fluoride-stimulated (▲) enzyme activities were measured in the absence of guanyl nucleotide (*A*) or in the presence of 100 μM GTP (*B*) or 100 μM Gpp[NH]p (*C*).

In contrast with the marked effects of Gpp[NH]p upon hormonal activation of adenylate cyclase in testis particles, the nucleotide had no effect on the binding properties of the testicular LH receptors. No changes in association and dissociation rate constants or in receptor affinity and binding capacity for hCG were observed when LH receptor assays were performed in the presence or absence of Gpp[NH]p and GTP.

Hormone-Dependent Binding of Guanyl Nucleotide. The role of guanyl nucleotides in LH action was further analyzed by studies on the properties and hormone-dependence of Gpp[NH]p binding to the putative nucleotide regulatory site in Leydig cell membranes. Analysis of Scatchard plots or saturation curves derived from equilibrium binding studies with [^{3}H]Gpp[NH]p showed no change in binding affinity for the nucleotide in the presence or absence of LH (1.5 ± 0.3 vs. 1.4 ± 0.4 × 10^7 M^{-1}; mean ± SEM). However, significant increases (2- to 3-fold) in binding capacity were observed; binding sites were 51 ± 4 and 117 ± pmol/mg in the absence and presence of LH, respectively, indicating that LH increases the number of available nucleotide binding sites (Fig. 3). Similar increases

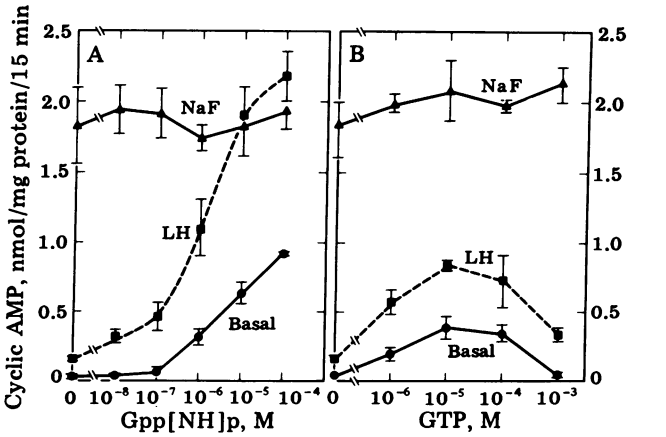

FIG. 2. Dose-dependent effects of Gpp[NH]p (*A*) and GTP (*B*) upon basal and hormone-stimulated adenylate cyclase activity in Leydig cell membranes. No significant change in fluoride-stimulated activity was observed in the presence of either nucleotide. Data are shown as mean ± SD for quadruplicate incubations. Incubations were for 15 min at 30°C.

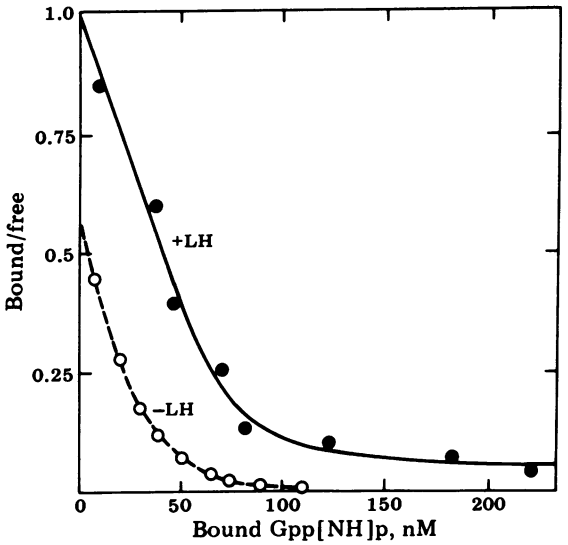

FIG. 3. Scatchard plot of [^{3}H]Gpp[NH]p binding to interstitial cell membranes incubated in the absence (○) or presence (●) of 30 μM ovine LH.

in nucleotide binding capacity were observed when the partially purified membrane fraction from a murine Leydig cell tumor was incubated with LH. In contrast, other pituitary hormones including thyrotropin, growth hormone, prolactin, and corticotropin did not produce the changes in nucleotide binding that were elicited by the homologous hormones, LH and hCG.

Although Scatchard plots of Gpp[NH]p binding sometimes showed the presence of a low-affinity site with $K_a = 10^5$ M^{-1}, the concentration of such sites was not significantly altered by hCG. Measurements showed twice as many low-affinity sites as high-affinity sites, but with relatively low precision that precluded the detection of minor changes in tissue concentration during hormone action. The presence of such low-affinity sites may reflect binding of nucleotide to nonregulatory membrane proteins and would account for the rapid early phase of Gpp(NH)p dissociation observed during kinetic analysis of nucleotide binding (below).

During association studies, Gpp[NH]p binding reached a steady state after 30 min, with a forward rate constant of 1.7×10^6 M^{-1} min^{-1}. In three experiments, the association rate constant was decreased by about 50% in the presence of LH, to 0.73×10^6 M^{-1} min^{-1} ($P < 0.05$). An increase of at least 2-fold in Gpp[NH]p binding at steady state was consistently observed in the presence of hormone (Fig. 4*A*). During dissociation studies, the release of bound Gpp[NH]p showed two major

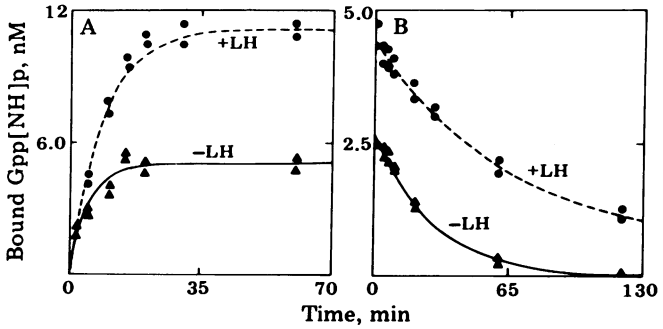

FIG. 4. Time course of association (*A*) and dissociation (*B*) of [^{3}H]Gpp[NH]p in Leydig cell membranes at 30°C, in the presence (●) and absence (▲) of LH.

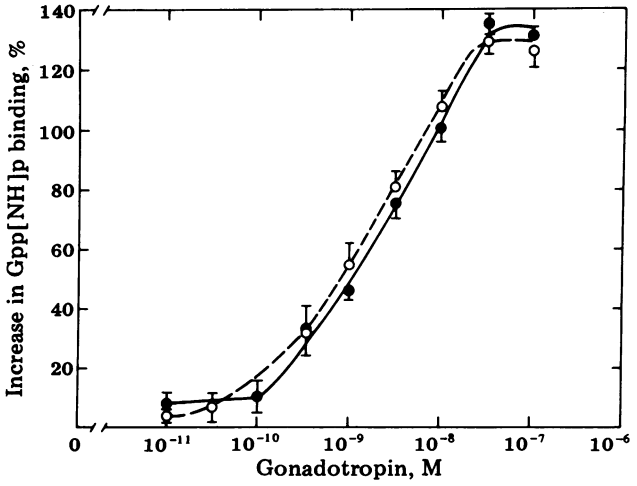

FIG. 5. Hormone-dependent increases in [^{3}H]Gpp[NH]p binding sites in Leydig cell membranes, shown as mean ± SD of triplicate incubations. ●, LH; ○, hCG.

components. About 50% of the sites dissociated very rapidly, with a rate constant of 1.0 min^{-1} (not shown). The remaining sites dissociated more slowly, with a rate constant of 3.4×10^{-2} min^{-1}. In the presence of LH, this component was even slower with a rate constant of 1.6×10^{-2} min^{-1}. The affinity constant (K_a) calculated from the kinetic properties of the second component was similar in the presence (4.6×10^7 M^{-1}) or absence (5.0×10^7 M^{-1}) of LH (Fig. 4*B*) and was consistent in magnitude with the association constant of 1.5×10^7 M^{-1} derived from equilibrium binding studies.

Correlation Between Hormone-Induced Nucleotide Binding and Activation of Adenylate Cyclase. The increase in guanyl nucleotide binding capacity induced by hCG was

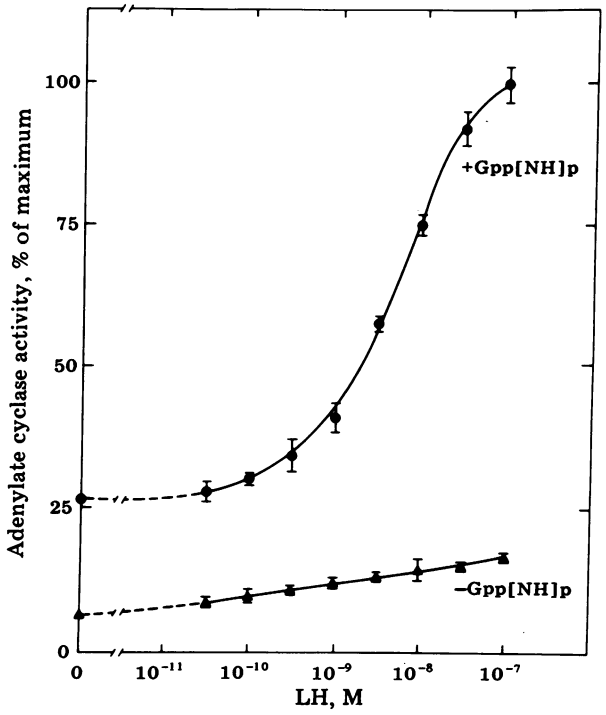

FIG. 6. Hormone-dependent increases in adenylate cyclase activity in the presence (●) or absence (▲) of 10 μM Gpp[NH]p during incubations at 30°C for 15 min, shown as mean ± SD of triplicate incubations.

dose-related over the range of 5 to 500 nM LH or hCG (50% effective dose, 50 nM hCG) (Fig. 5). In parallel studies on adenylate cyclase activation by hCG in the presence of 10 μM Gpp[NH]p, a significant increase above basal activity was observed at 5 nM hormone, with a progressive dose-related increase to a maximum at 100 nM hCG that was equal to the fluoride-stimulated activity (Fig. 6). The 50% effective dose of hCG for enzyme activation was 50 nM, similar to the concentration requirement for half-maximal stimulation of hormone-dependent nucleotide binding.

DISCUSSION

There is now abundant evidence for the importance of GTP-binding proteins in the regulation of adenylate cyclase by hormones and neurotransmitters. In many tissues, a GTP-binding membrane protein of M_r 42,000 has been found to mediate hormonal activation of adenylate cyclase. This action of hormones is believed to depend on binding of GTP to the nucleotide regulatory protein and to be terminated by hydrolysis of the bound nucleotide by a local GTPase reaction, with retention of tightly bound GDP at the regulatory site (20–22). The primary effect of hormonal ligands has been proposed to be exerted upon the exchange of guanyl nucleotides at the regulatory site, by enhancing the dissociation of bound GDP and facilitating its replacement by the activating nucleotide, GTP (21).

Although this mechanism has been supported by the demonstration of catecholamine-enhanced release of bound Gpp[NH]p from activated turkey erythrocyte membranes (22) and of GDP from membranes previously incubated with GTP and hormone (21), there has been no direct evidence for hormone-stimulated binding of guanyl nucleotide in membrane preparations from the target tissue. The major finding of the present study was the presence of high-affinity guanyl nucleotide binding sites in testicular interstitial cell membranes and their regulation by LH during hormonal activation of adenylate cyclase.

The dependence of Leydig cell adenylate cyclase on guanyl nucleotide was clearly indicated by the ability of GTP and Gpp[NH]p to amplify the hormonal stimulation of enzyme activity by LH or hCG. The inhibitory effect of high GTP concentrations was in contrast with the progressively greater effect of increasing concentrations of the nonhydrolyzable analog Gpp[NH]p. This effect of GTP could result from one of several mechanisms. First, excessive GDP formation and subsequent competition for bound GTP could occur, depending on the K_d for binding to the nucleotide regulatory protein and the K_m of the tissue nucleotide triphosphatases responsible for degradation of the higher concentrations of GTP. Because Gpp[NH]p is not hydrolyzed to GDP by tissue NTPases, it would not give rise to a competing species at higher concentrations and would progressively enhance the effect of hormone upon adenylate cyclase. Second, the decrease in enzyme activity at high GTP concentrations could reflect the role of a GTP-dependent phosphorylation in desensitization of adenylate cyclase (23), whereas Gpp[NH]p would not act as substrate for such a process. Third, the inhibitory actions of GTP observed in other tissues, such as the adipocyte (24, 25), may also occur in the Leydig cell as part of a bimodal regulation of adenylate cyclase in which as yet unidentified ligands could act to inhibit cyclic AMP production.

The LH-induced increases of high-affinity Gpp[NH]p binding observed in this study are consistent with the idea that hormone–receptor complex formation is followed by functional interaction with the nucleotide regulatory protein and consequent enhancement of GTP binding (26). This increase in binding sites could be attributed to exposure of hindered sites or, as recently proposed (26), to hormone-induced exchange of guanyl nucleotides with the species (probably GDP) that occupies the regulatory site. The latter effect would favor the binding of GTP or analogs such as Gpp[NH]p and would lead to concomitant activation of the catalytic subunit. The close correlation between hormone-stimulated nucleotide binding and activation of adenylate cyclase in the Leydig cell membranes observed in this study is consistent with the direct role of GTP binding in controlling the activity of the catalytic enzyme subunit during hormone stimulation.

The amount of Gpp[NH]p bound to Leydig cell membranes and regulated by LH was considerably greater than the number of LH/hCG binding sites (50 pmol/mg of protein vs. 0.8 pmol/mg of protein). This difference suggests that interaction of each hormone receptor with LH/hCG could activate a large number of nucleotide regulatory units, resulting in a 50-fold magnification of the gonadotropin-binding reaction. This is in contrast with the rather small fraction of guanyl nucleotide sites involved in catecholamine action, in which a close relationship between hormone–receptor complexes and nucleotide units has been proposed. The latter studies are based on indirect evidence of nucleotide release from regulatory sites under conditions in which hormone-induced dissociation of bound nucleotide can be evaluated (20–22).

Based on direct assay of nucleotide binding, our results indicate that each hormone–receptor complex could interact with a relatively large number of nucleotide regulatory units in the Leydig cell. The occurrence of such an amplification factor between hormone–receptor complex formation and nucleotide binding is relevant to recent observations on the existence of oligomers of the receptor-nucleotide protein that could allow maximal production of the activating nucleotide-GTP complex with minimal occupancy of hormone receptors (27). In relation to this model, the Leydig cell membrane could possess a more highly developed oligomeric receptor-nucleotide protein complex than that attributed to the liver, adipocyte, or erythrocyte adenylate cyclase system. The presence of a hormone-stimulated nucleotide binding process in Leydig cell membranes should permit more detailed clarification of the interactions between hormone–receptor complexes, guanyl nucleotides, and adenylate cyclase during the regulation of testicular function by gonadotropic hormones.

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