Physical and Functional Association of Follitropin Receptors with Cholera Toxin-sensitive Guanine Nucleotide-binding Protein*

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We have previously reported detergent (Triton X-100) solubilization of a follitropin (FSH) receptor-rich fraction from light membranes of bovine testis that responded to exogenous FSH by activation of adenylate cyclase (Dattatreymurty, B., Schneyer, A., and Reichert, L. E., Jr. (1986) J. Biol. Chem. 261, 13104–13113). Upon gel filtration of the detergent-extract through Sepharose-6B, two fractions were separated. Each specifically bound [32P]guanosine 5'-imidotriphosphate (Gpp(NH)p) and had guaninetriphosphatase (GTPase) activity. Of these, one fraction (6B-Fraction-1) also bound radiolabeled human follitropin (hFSH), indicating a coelution of the nucleotide-binding protein with receptor. The other fraction (6B-Fraction-2) did not contain detectable FSH receptor activity. Several lines of evidence suggest that 6B-Fraction-1 is a complex consisting of FSH receptor and a guanine nucleotide regulatory protein, probably N

FSH receptors are physically and functionally associated with a guanine nucleotide regulatory protein, probably N in light membranes of calf testis. Moreover, another component containing only high affinity binding sites for GTP represents a discrete molecular entity free from FSH receptors and is capable of hydrolysis of GTP. The availability of separate components having either high affinity FSH receptors associated with a guanine nucleotide regulatory protein or high affinity GTP-binding sites free of FSH receptors should facilitate further studies on molecular mechanisms of FSH activation of adenylate cyclase.

High affinity receptors for FSH1 present on testicular membranes (1–5) mediate several biochemical actions of FSH on testis (6–8). One of the early steps in FSH action is the stimulation of adenylate cyclase activity with a concomitant increase in cyclic AMP production (9–11). We have shown that rat testicular plasma membrane system responds to GTP and its analogues by increased adenylate cyclase activity in response to FSH (12). Moreover, specific high affinity binding sites for these nucleotides are shown to be present in Sertoli cell membranes (13). It has been speculated that the primary control for the stimulation of adenylate cyclase activity by FSH and guanine nucleotides is through interaction between FSH-occupied receptor and stimulatory guanine regulatory protein (N). No information, however, is available on the molecular mechanism of the FSH receptor-guanine nucleotide-binding protein interaction. Such studies require an approach involving the solubilization of FSH receptors, GTP-binding protein, and catalytic unit of adenylate cyclase in a functional state. Previous efforts in this regard have not been successful due to rapid destabilization of components of the adenylate cyclase system, once solubilized by detergents (3, 11, 14). In a recent report in this journal (1), we described a new protocol for solubilizing FSH receptors from lighter membranes of bovine calf testis. The detergent-soluble FSH receptor preparation so obtained retained receptor and guanine nucleotide-binding activities and responded to exogenous FSH by activation of adenylate cyclase. In this report we extend these studies to probe the molecular and functional relationship between FSH receptors and guanine nucleotide binding sites. Our present data suggest that FSH receptors

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1 The abbreviations used are: FSH, follitropin; [32P]-hFSH, radioiodinated human follitropin; Gpp(NH)p, guanosine 5'-imidotriphosphate; GTPase, guaninetrophosphatase; DT, dithiothreitol; App(NH)p, adenosine 5'-imidotriphosphate; N'-protein, guanine nucleotide-binding regulatory component of adenylate cyclase; N, stimulatory guanine nucleotide-binding protein of adenylate cyclase; DC-2-Fr II, concentrated light membranes of calf testis; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
are physically associated with a putative guanine nucleotide regulatory protein, probably *N*, a GTP-sensitive state. Moreover, we have observed a separate component having high affinity nucleotide-binding sites for GTP that is free from FSH receptors and capable of hydrolysis of GTP.

**EXPERIMENTAL PROCEDURES**

**Materials**

Calf testes were obtained from a local abattoir and kept at -20 °C until used. Na[32]P and [γ-32P]NAD were obtained from Du Pont-New England Nuclear. Lactoperoxidase, polyethylene glycol (PEG 8000), 5' -nucleotidase, alkaline phosphatase, thymidine, pyrophosphate, Triton X-100, ATP, ADP, ITP, and GDP were purchased from Sigma. p-Hydroxymercuribenzoate sodium was from Worthington. [3H]GPP(NH)p was from ICN Radiochemicals, Irvine, CA (γ-32P)GTP obtained from ICN was purified prior to use (see Methods).

Cholera toxin and pertussis toxin were purchased from List Biological Laboratories, Campbell, CA. The HI-50 hollow fiber cartridges, DC-10 and DC-2 filtration units were purchased from Amicon Corp, Lexington, MA. GTP, Gpp(NH)p, and App(NH)p were from Boehringer Mannheim. Ultrogel Aca-34 was from LKB Instruments, Gaitherburg, MD. GTP-Sepharose, Sepharose-4B, Sepharose-6B, and Sephadex G-100 were from Pharmacia.

**Methods**

**Preparation of Testicular Light Plasma Membranes**—FSH receptor-rich light plasma membranes were isolated and purified from 11.5 kg batches of bovine calf testes, according to a procedure previously described by us (1). These membranes (DC-2-Fr II) contained approximately 780 fmol of high affinity FSH receptors/mg of membrane protein and were stored at -80 °C until used.

**Solubilisation of FSH Receptors**—A previously described procedure (1) was employed to solubilize FSH receptors from light plasma membranes. In brief, membrane-enriched fraction (DC-2-Fr II) was suspended to a concentration of 50 mg of protein/ml in 10 mM Tris-HCl buffer, pH 7.2, containing 1 mM MgCl2, 0.001% NaN3, 0.004% 2-mercaptoethanol, and 40 μM p-hydroxymercuribenzoate sodium.

**Affinity Chromatography on GTP-Sepharose**—GTP-Sepharose derivative (Pharmacia) was washed with 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM MgCl2, 2.5% sucrose, and 0.05% Triton X-100 (buffer-A), packed by centrifugation, and resuspended in two times its volume with buffer-A. Aliquots of active fraction eluted from Sepharose-6B column (6B-Fr-1, see "Results") were added to two volumes of suspension (1.5 ml) of either Sepharose-4B (control) or GTP-Sepharose derivative. The mixture was shaken gently for 45 min at room temperature. Sample treatment with GTP-Sepharose was performed in the absence and presence of 250 μM of Gpp(NH)p. The reaction mixture was separated by centrifugation at 2000 × g for 10 min in the cold into supernatant (unretained material) and the matrix-bound materials. The GTP-Sepharose-bound material was washed twice with buffer-A and eluted by bringing the total volume to 2.25 ml with buffer-A containing 500 μM Gpp(NH)p. After 45 min of incubation with gentle shaking, the released proteins were separated by centrifugation of the supernatant. Binding and protein content of the matrix-bound materials were assayed for [32P]GTP binding and protein content.

**Characterization of 6B-Fr-1 and 6B-Fr-2 by Gel Filtration Chromatography**—6B-Fr-1 (2.5 ml) was preincubated with [32P]hFSH (60 ng) alone or with nonradioactive hFSH (1.000-fold molar excess) for 16 h in cold (4 °C). The incubation mixture was then applied on a column (1.75 × 85 cm) of Sepharose-4B equilibrated with 10 mM Tris-HCl buffer, pH 7.2, containing 1 mM MgCl2, 0.001% NaN3 and 0.0975% Triton X-100. The flow rate was 8.5 ml/h, and 2-ml fractions were collected and assayed for [32P]radioactivity. Preincubation of 6B-Fr-1 with [32P]hFSH in the absence or presence of 6B-Fr-1 bound [32P]hFSH peak. Similarly 6B-Fr-2 (1 ml) was preincubated with [3H]Gpp(NH)p (150 pmol) alone or with nonradioactive Gpp(NH)p (50 μM) for 8 h at 4 °C and subjected to gel filtration on Sepharose-4B column under identical conditions. Fractions were collected and assayed for [3H]radioactivity. Preincubation of 6B-Fr-2 with [3H]Gpp(NH)p in the presence of nonradioactive Gpp(NH)p eliminated 6B-Fr-2-bound [3H]Gpp(NH)p peak. The column was calibrated with protein markers, thyroglobulin, ferritin (M, = 440,000), catalase (M, = 232,000), and alcohol dehydrogenase (M, = 150,000). Void volume of the column was determined by using blue dextran. Molecular weight estimates of active fractions (6B-Fr-1 and 6B-Fr-2) were made by interpolation, using the linear relationship between the distribution coefficient and log molecular weight of the standard proteins.

**Preparation of FSH-Receptor Complex**—Five ml of 6B-Fr-1 (FSH receptor-containing fraction) was preincubated with 100 ng of [32P]hFSH for 16 h at 4 °C. The receptor (6B-Fr-1) bound hormone was separated from free hormone by gel filtration on a column (2.5 × 35 cm) of Ultrogel Aca-34 equilibrated with 10 mM Tris-HCl buffer, pH 7.2, containing 1 mM MgCl2, 0.001% NaN3, and 0.0975% Triton X-100. Fractions of 2 ml were collected and assayed for bound activity in a gamma counter. Fractions collected in void volume were pooled and used immediately in incubation experiments with GTP/other nucleotides. Preincubation of 6B-Fr-1 with [32P]hFSH in the presence of nonradioactive hFSH (1000-fold molar excess) eliminated the radioactive peak (receptor-bound [32P]hFSH) in the void volume.

Experiments to examine the effects of GTP on [32P]hFSH binding to FSH receptors were carried out under standard assay conditions (1). Aliquots of 6B-Fr-1 (60-150 μg of protein) were incubated with approximately 2.5 ng of [32P]hFSH in the absence or presence of 10 μM GTP in 1 ml of 40 mM Tris-HCl buffer, pH 7.1, containing 1 mM MgCl2, 1 mM ATP, 100 μg of BSA, and 1 mM DTT for 16 h, at 4 °C. Receptor-bound [32P]hFSH was separated from free hormone by PEG precipitation as described earlier (1). The radioactivity in the pellet, representing [32P]hFSH-receptor complex was counted in an autogamma counter with an efficiency of 75% for [32P].

**Treatment of Membranes with Cholera Toxin or Pertussis Toxin**—Treatment of testis membranes with activated pertussis or cholera toxin was performed according to the procedures of Lin et al. (15) and Kataoka and Uli (16). Highly pure testis membranes (5 mg of protein/ml) were incubated with 25 μg of activated pertussis toxin at 30 °C for 15 min in 1 ml of 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM MgCl2, 10 μM NaF, and 0.0975% Triton X-100, 1 mM DTT. Controls were performed without treatment with toxin or without NAD. Treated membranes were washed twice with 25 mM Tris-HCl, pH 7.5, containing 2.5 mM MgCl2, and then with 10 mM Tris-HCl, pH 7.2, containing 1 mM MgCl2 and 0.001% NaN3. [32P]hFSH binding to receptors in these membranes, and subsequent dissociation of bound [32P]hFSH from membranes, in the presence and absence of GTP were assayed as described above.

**Cholera Toxin-catalysed ADP-ribosylation of Membranes**—Cholera toxin (2.5 mg/ml) was activated before use by incubation for 10 min at 37 °C in 25 mM Hepes, pH 7.5, containing 0.5% sodium dodecyl sulfate, 5 mM DTT, and 0.1% BSA, as described (17). For ADP-ribosylation, highly pure testis membranes (7 mg of protein/ml) were incubated with activated toxin (250 μg/ml) in 40 mM Tris-HCl buffer, pH 7.5, containing 2.5 mM MgCl2, 0.1% BSA, 10 mM sodium thymidine, and 1 mM DTT. Controls were performed without treatment with toxin or without NAD. Treated membranes were washed twice with 25 mM Tris-HCl, pH 7.5, containing 2.5 mM MgCl2, and then with 10 mM Tris-HCl, pH 7.2, containing 1 mM MgCl2 and 0.001% NaN3. [32P]hFSH binding to receptors in these membranes, and subsequent dissociation of bound [32P]hFSH from membranes, in the presence and absence of GTP were assayed as described above.
According to the method of Lowry et al. (18) with minor modifications.

Gel filtration through a column of Sephadex G-100 or disc gel electrophoresis (21) to separate radioiodinated hormone from free iodide. The specific activity of the 125I-FSH, as determined by self displacement (22), was 22-31 μCi/μg. The percent bindability of radioiodinated preparations to excess receptor was 25-32%.

Detergent-extracted receptor preparations and column effluent fractions were assayed for 125I-FSH-binding activity, as described earlier (1). The quantitative determination of FSH-binding capacity was carried out by equilibrium with approximately 2.5 ng of FSH tracer, in the presence of increasing concentrations of unlabeled FSH (LER 2050-3A, 1 to 10,000 ng). The assay procedure was the same as described earlier (1). The affinity constant and FSH receptor concentration were estimated from competitive data using the LIGAND program of Munson and Rodbard (23).

To determine the 5'-nucleotidase activity, the method of Windall and Unkeless (24) was employed with minor modifications (25). The results were expressed as micromoles of inorganic phosphate generated per hour per milligram of protein.

Binding of 3H]Gpp(NH)p to detergent-extracted preparations and column effluent fractions was determined according to the method of Pfeuffer and Helmerich (26), with some modifications as described earlier (1).

GTPase Assay—The GTPase assay was performed as described by Cassel and Selinger (27), but modified by Snyer et al. (28). The final concentrations in the reaction medium consisted of 25 mM Tris-HCl buffer, pH 7.5, 1 mM MgCl2, 1 mM EDTA, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase (200 units/mg), and 1 mM cAMP. Labeled GTP substrate was repurified according to the method of Iyengar and Birnbaumer (29), aliquoted, and stored frozen at -20°C. Ten to 20 μg of sample protein were incubated with 25 nM GTP[y-32P] (100,000-400,000 cpm) in 100 μl of above reaction medium for 4 min at 30°C. Parallel set of tubes also received [NH]p and ATP, according to the assay procedure. App[NH]p, an inhibitor of α number of ATPases (30, 31), when added to the assay, was at 6.4 μM to decrease the rate of GTP hydrolysis by nonspecific NTPases (27). Moreover, the suppression of the transfer reaction caused by a nucleotide triphosphate regeneration system was further enhanced by the addition of 1 mM ATP providing a greater specificity to the assay.

The assay reaction was stopped by immediately placing the tubes on ice and adding 0.75 ml of ice-cold stopping solution (20 mM phosphoric acid, pH 2.1, containing 1 mM MgCl2, 0.001% 2-mercaptoethanol, and 0.1% Triton X-100 as described under Methods, and approximately 185 mg of detergent-extracted preparations/column effluent active fractions were pooled and used for further study.

RESULTS

Gel Filtration on a Sepharose-6B Column—Fractionation of Triton X-100-soluble light plasma membrane protein in aliquots of 185 mg, on a column of Sepharose-6B (71 × 2.8 cm) resulted in two major fractions (Fig. 1A). Sepharose-6B-Fraction-1 (6B-Fr-1) representing column effluent fractions 72-100 contained FSH-binding activity. Some Gpp(NH)p-binding activity was also coeluted in this fraction. Most Gpp(NH)p-binding activity was, however, eluted in more retarded Sepharose-6B-Fraction-2 (6B-Fr-2) (column effluent fractions 105-140) and was clearly separated from the hormone-binding activity. The peak 5'-nucleotidase activity was eluted in the void volume (Fig. 1B), emerging prior to hormone-binding activities.

To ensure that fractions 6B-Fr-1 and 6B-Fr-2 were free of contamination from one another, they were concentrated 2.5-fold by ultrafiltration, and then each fraction was reconceptuated through the same column of Sepharose 6B. Only tubes about the apex region of elution profile for each fraction were pooled and used for further study.

Further characterization of these fractions by chromatography through Sepharose-4B indicated that both active fractions 6B-Fr-1 and 6B-Fr-2 were eluted as retarded discrete peaks with Kao estimates of 0.402 (Md > 669,000) and 0.623 (Md = 205,000), respectively.

Affinity Chromatography on GTP-Sepharose—When 6B-Fr-1 was incubated with GTP-Sepharose approximately 85% of FSH-binding activity was retained by the affinity matrix. About 82% of the bound receptor was eluted by 0.5 mM Gpp[NH]p. When affinity chromatography was carried out in the presence of 250 μM Gpp[NH]p (which effectively competes with Sepharose-bound GTP for the N-protein), the affinity matrix failed to retain significant FSH receptor activity. In control experiments, undervatized Sepharose-4B failed to retain significant receptor activity. These results suggest that the FSH receptors and Gpp(NH)p-binding component coeluted in 6B-Fr-1 were physically associated.

It was of interest, therefore, to know if FSH receptors and Gpp[NH]p-binding component present in 6B-Fr-1 were functionally related. To this end, we studied the FSH receptor sensitivity to guanine nucleotides, a heterotropic property which previously has been well-characterized in several other receptor systems, as an evidence of functional association between receptor and guanine nucleotide-binding protein (32-35).

Effect of GTP on 125I-FSH Binding to 6B-Fr-1—When 125I-FSH and increasing concentrations of GTP were simultaneously incubated with 6B-Fr-1, GTP effectively inhibited the
binding of FSH tracer to 6B-Fr-1 in a dose-dependent manner (Fig. 2A). The slopes of the dose response lines for GTP (−3.28) and unlabeled FSH (−1.85) were different (Fig. 2B). Here the slope represents the fall in the percent bound (expressed as probits) per log dose of FSH or GTP. The concentration of GTP, on a mass basis, required to cause 50% inhibition of 125I-FSH binding was 92-fold higher than that of unlabeled FSH. Moreover, the interactions of GTP and FSH appear to be noncompetitive at FSH-binding site, since a higher occupancy of receptors with 125I-FSH (close to saturation) did not decrease the GTP effect on hormone binding.

Kinetics of 125I-FSH Binding as Influenced by GTP—To ascertain the mechanism of the GTP effect on 125I-FSH binding, we examined the kinetics of 125I-FSH binding to 6B-Fr-1 (Fig. 3). In the absence of GTP, specific 125I-FSH binding was saturable. Scatchard analysis indicated a single class of high affinity FSH-binding sites with a dissociation constant (Kd) of 0.31 nM and a maximal number of binding sites (Bmax) of 4530 fmol/mg. On the other hand, the presence of GTP at 0.5 μmol/assay tube reduced receptor affinity from 0.31 to 1.49 nM without affecting the Bmax value. Thus, it appears that GTP reduces 125I-FSH binding predominantly by decreasing the apparent affinity of binding sites for FSH.

Nucleotide Specificity for Dissociation of Bound 125I-FSH from 6B-Fr-1—FSH-Receptor complex was prepared by incubating 125I-FSH with 6B-Fr-1, in the cold (4°C) for 16 h, and by separating the unbound 125I-FSH by filtration through Ultrogel AcA-34 column. Aliquots of the FSH-receptor complex were then incubated with increasing concentrations of various nucleotides for 15 min at 30°C to study dissociation of specifically bound 125I-FSH. In a parallel experiment, a 125I-FSH tracer was rapidly released by the addition of excess unlabeled FSH (4 μg/tube) to the incubation medium (Fig. 3). GDP required 9.2-fold higher concentration than GTP. The relative potencies of ATP, ADP, and AMP were less than 5% which can be attributed to the known contamination of guanine nucleotide in these commercial preparations. UTP, ITP, App(NH)p, CAMP, cGMP, and GMP were ineffective. Gpp(NH)p, a nonhydrolyzable analogue of GTP mimicked the GTP effect, but required 15 times higher concentration than that of GTP. It has also been shown in previous studies that concentrations similar to, or greater than, this were required by Gpp(NH)p to influence the binding of other hormones that stimulate adenylyl cyclase (36–37).

Effect of GTP on Time Course of Dissociation of Bound 125I-FSH—The effect of GTP (1 μmol/assay tube) on dissociation of FSH bound to receptor in 6B-Fr-1 was rapid in onset. A significant dissociation was observed within 1 min of the addition of GTP to the incubation medium (Fig. 4A). In the absence of GTP, no significant dissociation of bound 125I-FSH was observed after 60 min of incubation. The addition of excess unlabeled FSH (4 μg/tube) to the incubation medium did not influence the rate of dissociation of 125I-FSH in either the absence or presence of GTP.

Effect of GTP on Gel Filtration Profile of 125I-FSH-Receptor Complex—In previous experiments, the modulation of FSH binding to receptor by GTP was shown indirectly based on the failure of 125I-hFSH to coprecipitate with receptor during PEG separation. The present experiment was carried out to demonstrate directly that 125I-hFSH was released as free hormone from receptor-hormone complex in response to GTP effect. 125I-FSH-Receptor (6B-Fr-1) complex was prepared and separated from unbound 125I-FSH by gel filtration as described under “Methods.” Equal aliquots of FSH-receptor complex were then incubated in the absence or presence of GTP (1 μmol/assay tube) for 10 min, at 30°C. Following incubation, they were fractionated on a column of Ultrogel AcA-34. Gel filtration profile of the hormone-receptor complex incubated in the absence of GTP revealed a single major peak emerging in the void volume. On the other hand, the profile of hormone-receptor complex after incubation with
Follitropin Receptors in Relation to GTP-binding Sites

GTP showed a marked decrease in radioactivity in this peak with a concomitant increase in free 125I-FSH (Fig. 5).

Additional evidence to indicate that FSH receptor-associated GTP-binding activity was involved in GTP regulation of hormone binding to FSH receptor in 6B-Fr-1 was obtained as follows. We exposed Sepharose 6B-Fr-1 to a higher concentration of Triton X-100 (0.4%), while maintaining an appropriate ratio between sample protein and Triton X-100 previously shown to preserve receptor activity (1). Detergents at sufficiently elevated concentrations are known to uncouple GTP action. These results suggest that the GTP-sensitive FSH receptor activity is associated with N1, but not N2.

The likelihood that the nucleotide regulatory protein N1 was in association with the FSH receptor was further investigated as follows. Purified bovine testis membranes were treated with cholera toxin in the presence of [32P]NAD under conditions in which the N1 protein is specifically labeled by ADP-riboylation of its 5'-subunit (42-45). Membrane proteins extracted with Triton X-100 were then chromatographed on a Sepharose-6B column. A significant peak of 32P radioactivity was eluted after void volume. In the presence of cholera toxin pretreatment, this peak was very small (Fig. 6). Comparison of the elution profiles of membrane protein labeled either by [32P]ADP-riboylation or prebound cholera toxin plus NAD, completely eliminated GTP effect on FSH binding to its receptors (Table I). In contrast, treatment of membranes with cholera toxin plus NAD and washed extensively, total 125I radioactivity in column effluents was assessed, and recovery of 125I radioactivity exceeded 80% of that applied.

Fig. 5. Effect of GTP on the gel filtration profile of 125I-FSH-6B-Fr-1 complex. Aliquots of complex (approximately 10,000 cpm) were incubated, either at 30°C for 15 min or at 4°C for 16 h, in the presence (●) or absence (▲) of GTP (1 µmol/assay tube) and fractionated on a column of Ultrogel AcA-34 presaturated with 2% BSA and washed extensively. Total 125I radioactivity in column effluents was assessed, and recovery of 125I radioactivity exceeded 80% of that applied.
Effects of cholera toxin and pertussis toxin treatments on GTP effect on 125I-hFSH binding to its receptors in pure membranes of bovine testis

Bovine testis membranes were subjected to the indicated treatments and then incubated with 125I-hFSH to prepare hormone-receptor complexes, as described under Methods. The complexes were incubated in the presence or absence of GTP (2.5 μmol/ml), as described earlier (legend for Fig. 5) for 15 min at 30 °C. This experiment was performed twice with similar results.

| Treatment          | Percent specific binding of 125I-hFSH* |
|--------------------|--------------------------------------|
|                    | −GTP | +GTP |
| None               | 100.0 ± 6.02 | 22.7 ± 1.05 |
| NAD                | 100.0 ± 0.89 | 24.4 ± 1.11 |
| Cholera toxin      | 106.0 ± 4.99<sup>a</sup> | 21.9 ± 1.2 |
| Pertussis toxin    | 102.0 ± 2.15<sup>b</sup> | 20.7 ± 2.78 |
| Cholera toxin + NAD| 30.6 ± 2.10<sup>d</sup> | 32.2 ± 2.31<sup>f</sup> |
| Pertussis toxin + NAD| 112.0 ± 6.78<sup>e</sup> | 21.3 ± 1.07 |

*Values are mean ± S.D.
<sup>a</sup> p < 0.01.
<sup>b</sup> p > 0.05.
<sup>c</sup> p > 0.05.

Fig. 6. Comparison of elution profiles of solubilized membrane proteins preloaded with 125I-hFSH or [32P]ADP-ribosylated by cholera toxin (C-T). Pure testis membranes (7.5 mg of protein/ml) were incubated in the presence of [32P]NAD with (●) or without (O) activated cholera toxin (750 μg/ml), solubilized with 0.15% (v/v) Triton X-100 (final concentration), and chromatographed on Sepharose-6B column. The radioactivity eluted was determined. In parallel experiments, testis membranes were incubated with 125I-hFSH, solubilized, and applied on Sepharose-6B column. ▲ represents the elution profile of 125I-hFSH bound to soluble receptors.

Gpp(NH)p-binding Properties of FSH Receptor-associated and Receptor-free Fractions—Specific binding of [3H]Gpp(NH)p to fractions 6B-Fr-1 (FSH receptor-associated) and 6B-Fr-2 (receptor-free) were determined by Scatchard analysis. Differences exist in their [3H]Gpp(NH)p-binding properties. The Scatchard plot for the nucleotide-binding component in 6B-Fr-1 indicated the presence of low affinity (9.2 × 10<sup>-10</sup> M) and high capacity binding sites, while 6B-Fr-2 showed the presence of only high affinity (1.37 × 10<sup>-7</sup> M) and low capacity binding sites (Fig. 7). Importantly, the higher nucleotide concentration required by GTP for its effect on FSH binding to 6B-Fr-1 is compatible with the observed low affinity and high capacity binding sites present in this fraction.

The binding of [3H]Gpp(NH)p in both 6B-Fr-1 (receptor-associated) and 6B-Fr-2 (receptor-free) displayed a high degree of specificity. GTP at concentrations of 2.5 × 10<sup>-m</sup> M and 0.8 × 10<sup>-m</sup> M were required to inhibit 50% of total [3H]Gpp(NH)p-bound to 6B-Fr-1 and 6B-Fr-2, respectively. ATP, App(NH)p, ADP, and AMP failed to inhibit the binding of tracer to both fractions (Fig. 8). Thus, the presence of highly specific Gpp(NH)p/GTP-binding sites in these fractions indicate a higher degree of specificity in GTP interactions with these fractions, particularly 6B-Fr-1, while modulating FSH binding to its receptors.

A recent report (46) on β-adrenergic receptor system has suggested that enhanced GTPase activity in N<sub>e</sub> following its interaction with receptor provides a sensitive monitor for receptor-N<sub>e</sub> coupling. It was, therefore, of interest to compare GTPase-specific activities of 6B-Fr-1 and 6B-Fr-2. Each fraction contained GTPase activity. 6B-Fr-2 was 2–3 times less active than 6B-Fr-1 (Table II). The GTPase activity could be...
Tide regulatory protein, probably N., are physically associated, binding sites with a high affinity for Gpp(NH)p representing 20 mM phosphoric acid, pH 2.1, containing 5% (w/v) activated charcoal. ["Pi released was separated and counted thereafter. Specific activity (pmol Pi/mg of protein/min). Results are expressed as a percent of [3H]Gpp(NH)p bound in the presence of various nucleotides for 120 min at 30 °C. Separation of either fractions (40 µg of protein) were incubated with 20 pmol of [3H]Gpp(NH)p in the presence or absence of indicated concentrations of various nucleotides for 120 min at 30 °C. Separation of bound and free-labeled Gpp(NH)p was accomplished as described earlier (1). Results are expressed as a percent of [3H]Gpp(NH)p bound in the absence of nucleotides. Each point is the mean of triplicates that agree within 5% of the mean value.

The physically associated soluble FSH receptors and guanine nucleotide-regulatory protein, probably N, which is consistent with the ability of FSH to stimulate testicular adenylate cyclase (9-11). Exposure of these receptors with digitonin in the absence of ligand (hormone) was present during solubilization (47-49). Recent studies have shown that the a-subunit of N, regulatory protein is the site of cholera toxin-induced ADP-ribosylation (42-45). We infer, therefore, that the Triton-solubilized FSH receptors are associated with a guanine nucleotide regulatory protein, probably N, which is consistent with the ability of FSH to stimulate testicular adenylate cyclase (9-11). 5 Examination of differential effects of cholera toxin (plus NAD) and pertussis toxin (plus NAD) on GTP regulation of ligand binding also provided some insight about the putative guanine nucleotide regulatory protein associated with the FSH receptor. Pretreatment of the pure testis membranes with cholera toxin plus NAD, but not pertussis toxin plus NAD, completely eliminated GTP effect on FSH binding to its receptors. These results are consistent with the concept that GTP-sensitive, FSH receptor activity is associated probably with N, but not N.

There are several precedents for the solubilization of hormone receptor-N-protein complexes (47-51). In the earliest of these reports, complex formation occurred only when the ligand (hormone) was present during solubilization (47-49). More recently, A, adenosine receptor-N, complex (50) and prostaglandin-E, receptor-N, complex (51) were solubilized with digitonin in the absence of ligand.

In a previous study, it has been suggested that the detergent deoxycholate mimics nonspecifically the function which is normally fulfilled by the guanine nucleotide-binding protein in inducing the high affinity state of the b-adrenergic receptor (52). In the present study, the high affinity state of soluble FSH receptors in the absence of GTP cannot be attributed to similar effect, if any, of Triton X-100. This is because the ability of detergent to induce receptor affinity is unique to deoxycholate and is not common to all detergents (52). Control experiments employing Triton X-100 ruled out such a possibility in the present study. Moreover, we noticed that FSH receptor activity is more labile in the presence of in-

shown to be specific in both the fractions for guanosine-5'-triphosphate by the simultaneous addition of 1.0 mM ATP or 0.4 µM App(NH)p.

DISCUSSION

The present report provides the first evidence for a physical and functional association between unoccupied FSH receptors and a guanine nucleotide regulatory protein, probably N. We have also observed a separate pool of guanine nucleotide-binding sites with a high affinity for Gpp(NH)p representing a discrete molecular entity free from FSH receptor and in a GTP sensitive state. These findings are consistent with, and extend, our previous observations that GTP and its analogues can act either alone or with FSH to stimulate testicular adenylate cyclase activity (12).

Our conclusion that FSH receptors and a guanine nucleotide regulatory protein, probably N, are physically associated, is based on several lines of evidence. 1) Detergent-soluble FSH receptors and Gpp(NH)p-binding sites coeluted from Sepharose-6B column, and most FSH receptors and GTP-binding activity were retained by a GTP-Sepharose affinity matrix. Their retention by the affinity matrix could be prevented by simultaneous addition of free Gpp(NH)p, indicating that associated guanine nucleotide-binding protein was involved in FSH receptor retention by the affinity matrix. 2) The physically associated soluble FSH receptors and guanine nucleotide-binding protein are functionally related. This is shown by the negatively cooperative binding interactions of guanine nucleotide and FSH. In the absence of GTP, FSH bound with high affinity; whereas, in the presence of GTP, the binding of FSH to receptors decreased in a dose-dependent manner. Analysis of equilibrium-binding studies suggested that the decreased binding could not be accounted for by a decrease in the number of receptors per se. Rather, the altered binding isotherm was the result of a decrease in affinity of receptors for FSH. Also, an increase in dissociation rate prompted by GTP was reflected in significant decrease in FSH receptor affinity. Thus, in the absence of exogenous GTP, no significant dissociation of bound [32P]-FSH was observed even after 1 h, whereas in the presence of this agent, the dissociation was rapid (less than 1 min). 3) Detergents at sufficiently elevated concentrations are known to uncouple the functional units of adenylate cyclase (38, 39). Exposure of 6B-Fraction-1 to higher concentration of Triton X-100 in the present study reduced significantly the receptor-associated GTP-binding activity. Importantly, this also rendered FSH-binding to its receptor insensitive to GTP effect. 4) After cholera toxin-induced [32P]ADP-ribosylation of testis membranes, a high peak of [32P]radioactivity coeluted with FSH receptor activity from Sepharose-6B column. Recent studies have shown that the a-subunit of N, regulatory protein is the site of cholera toxin-induced ADP-ribosylation (42-45). We infer, therefore, that the Triton-solubilized FSH receptors are associated with a guanine nucleotide regulatory protein, probably N, which is consistent with the ability of FSH to stimulate testicular adenylate cyclase (9-11). 5 Examination of differential effects of cholera toxin (plus NAD) and pertussis toxin (plus NAD) on GTP regulation of ligand binding also provided some insight about the putative guanine nucleotide regulatory protein associated with the FSH receptor. Pretreatment of the pure testis membranes with cholera toxin plus NAD, but not pertussis toxin plus NAD, completely eliminated GTP effect on FSH binding to its receptors. These results are consistent with the concept that GTP-sensitive, FSH receptor activity is associated probably with N, but not N.

There are several precedents for the solubilization of hormone receptor-N-protein complexes (47-51). In the earliest of these reports, complex formation occurred only when the ligand (hormone) was present during solubilization (47-49). More recently, A, adenosine receptor-N, complex (50) and prostaglandin-E, receptor-N, complex (51) were solubilized with digitonin in the absence of ligand.
Increased concentrations of Triton X-100 (1), and an appropriate ratio of protein to detergent is critical in maintaining receptor activity after chromatography on Sepharose-6B. Through a series of chromatographic profiles of receptor activity, we established an optimum level of detergent relative to sample protein, which we used for column fractionation (as indicated under "Methods").

We have recently shown the presence of specific binding sites for Gpp(NH)p/GTP in large excess to FSH receptors in rat Sertoli cells (13). The specific binding of Gpp(NH)p/GTP to bovine testicular membranes represent two classes of binding sites, i.e., high affinity and low affinity, and low affinity and high affinity. Interestingly, the present studies indicate that FSH receptors exist in a high affinity state, independent of their association with high affinity GTP-binding sites.

Another noteworthy result of the present studies is the observation of significant GTPase activity in both FSH receptor-associated and receptor-free guanine nucleotide-binding components in the absence of hormone. Recently Cerione et al. (46) suggested that an induction of GTPase activity in guanine nucleotide-binding protein (N) following its interaction with β-adrenergic receptor provides a sensitive monitor for β-adrenergic receptor-N coupling. This was based on their observation that when large amounts of β-adrenergic receptor and N, were inserted into phospholipid vesicles, a significant induction of GTPase activity was noticed even in the absence of hormone. Our observation that the FSH receptor-associated guanine nucleotide component has higher GTPase activity than that of receptor-free component is consistent with their findings.

The present studies provide evidence for a physical association between FSH receptors and a guanine nucleotide regulatory protein, probably N, Our results (a significant decrease in FSH receptor affinity in response to GTP effect and loss of sensitivity to GTP in FSH binding after cholera toxin treatment of bovine testis membranes) suggest that GTP interaction with a guanine nucleotide regulatory protein may lead to the conformational change of FSH receptor protein, through a functional association between these two proteins. These results, together with the finding that FSH receptor interactions induce adenylate cyclase with concomitant increase in CAMP in bovine testis membranes (9-11), raise the possibility for a bidirectional coupling of N, to adenylate cyclase and FSH receptor. The availability of two separate components having either high affinity FSH receptors or high affinity GTP-binding sites should facilitate further studies on the molecular mechanisms of FSH activation of adenylate cyclase.

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