CHARACTERIZATION OF A SOLUBLE FOLLITROPIN RECEPTOR FROM PORCINE TESTIS

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SUMMARY.

Porcine testis receptors for follitropin (FSH) were solubilized by treatment with the non-ionic detergent Nonidet P-40 and receptor-bound and free ^{125}I-porcine FSH were separated by ammonium sulfate precipitation. The soluble receptor retained both its high affinity and specificity for FSH. The soluble hormone-receptor complex exhibited an equilibrium association constant of 4.7×10^{10} M^{-1} at 4°C. Its hydrodynamic properties were consistent with those obtained for other solubilized peptide hormone receptors, and its molecular weight estimated to 244,000.

INTRODUCTION.

Specific and high affinity receptors for LH/HCG and FSH have been demonstrated in the testis. The physicochemical properties of these receptors have been studied using particulate receptor preparations (1, 2,3,4). The LH/HCG receptors from rat testis have been further studied after solubilization by Triton X-100 (5,6) and have been partially purified (7).

In contrast, the receptor for FSH has not yet been characterized in its solubilized form. In this report we present data on the solubilization of FSH receptor from porcine testis by the use of the non-ionic detergent Nonidet P-40 (N.P-40). The binding characteristics and the physical properties of the soluble FSH receptor are presented and compared to those of the particulate receptor (8).

MATERIAL

Porcine FSH (82 times NIH-FSH-P1) and its subunits, porcine LH (1 time NIH-FSH-S9) and porcine TSH (25 UI/mg) were prepared in our laboratory (9,10,11). N.P-40 was obtained from BDH, Carrier Free ^{125}I from Amersham, Sepharose 4B - Concanavalin A, Sepharose 6B, Sephadex G-200, Blue Dextran were from Pharmacia Fine Chemicals ; Bovine Serum Albumin from Armour ; bovine γglobulins, human γ-globulins, Triton X-100, polyethyleneglycol 6000 from Koch-Light Laboratories ; Lithium diiodosalicylate from Eastman Kodak ; sodium deoxycholate, neuraminidase, phospholipase A (Vipera Russilii), phospholipase C (Clostridium Perfringens) from Sigma.

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METHODS.

Preparation of ^{125}I-pFSH.

Purified pFSH (82 times NIH-FSH-P1) was labeled with ^{125}I (Amersham) and purified by Sepharose-Concanavalin A chromatography as previously described (3). The specific activity of the labeled preparation as determined by self displacement method (17) was of 70 to 100 μ Ci/μg and 35 % of the tracer reacted specifically with an excess of particulate binding sites.

Solubilization of testis particules.

The experiments were conducted at 2-4°C. Particulate fraction (10 mg of protein/ml) prepared by homogeneization of immature porcine testis and centrifugation at 27,000 x g as previously described (5,8) was washed twice with 6 ml of 0.05 M Tris-HCl buffer pH 7.4 and centrifuged. The pellet was resuspended in 6 ml of 1 % N.P-40 at 1 % final concentration and homogenized. After 1 hour, the suspension was centrifuged at 27,000 x g for 20 min. The supernatant was then submitted to centrifugation at 300,000 g for 1 hour. The resulting supernatant was defined as our solubilized FSH receptor preparation. Similar procedures were followed for the extraction of prelabeled hormone-receptor complex obtained after 20 hoursincubation of ^{125}I-pFSH (2 x 10^6 cpm) with 2 ml of the original particulate fraction at 4°C.

Assay for receptor binding.

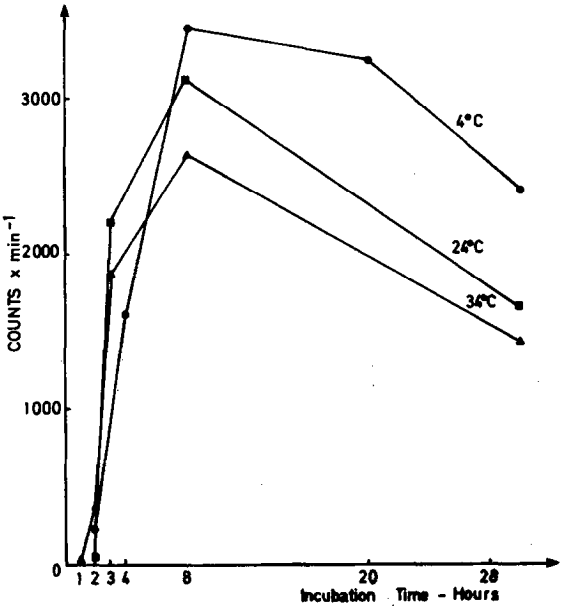
Aliquots of the receptor solution (0.5 ml of the 300,000 g supernatant) were mixed with 0.1 ml Tris buffer, pH 7.4 containing no pFSH or known quantities of unlabeled pFSH and 0.1 ml of ^{125}I-pFSH in Tris chloride buffer, pH 7.4 (25.000 cpm). Non-specific binding was determined from tubes containing labeled hormone and receptor in the presence of an excess (640 ng) of unlabeled pFSH. After incubation for 16 hours at 4°C 200 μl of a bovine γ-globulin solution (10 mg/ml Tris buffer) and 1.6 ml of 2 M ammonium sulfate were added. After mixing, the tubes were centrifuged at 2,000 x g for 15 min and the supernatant was discarded by aspiration. The bound hormone was determined by counting the radioactivity of the precipitates in a gamma-spectrometer. The non-specific binding was usually around 5 % of the total radioactivity. Alternatively, polyethyleneglycol precipitations were assayed in the conditions described by Dufau et al (5) to separate bound and free hormones.

Gel filtration.

Chromatographies on Sepharose 6B and Sephadex G-200 were performed on calibrated columns in the conditions described under fig. 4. Values for K_{av} of the hormone-receptor complex and the free hormone were determined according to Laurent and Killander (12).

Density gradient centrifugation.

Centrifugation in continuous 5 to 20 % (W/V) sucrose gradient in 50 mM Tris-HCl buffer, pH 7.4 containing 0.1 % N.P-40 were performed in a Beckman L-2 ultracentrifuge according to Dufau et al (5). The density of the hormone-receptor complex and free hormone were determined by isopycnic density gradient of cesium chloride in 50 mM Tris-HCl buffer, pH 7.4 containing 0.1 % N.P-40 in the conditions described by Dufau et



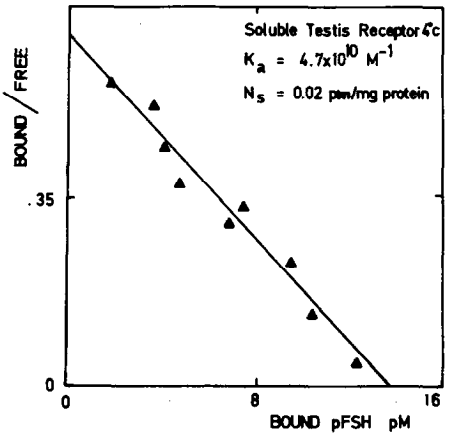


Figure 1. Association time course of ^{125}I-pFSH with soluble receptors during incubation at 4°, 24° and 34°C for 28 hours. Non-specific control levels for each time point were determined by addition of 640 ng of pFSH and deduced from each value.

Figure 2. Scatchard plot derived from the binding inhibition experiment of ^{125}I-pFSH by native pFSH at 4°C. "N_s" **number of specific sites**

al (5). Density of collected fractions were measured using an Abbe refractometer. The molecular weight of the hormone-receptor complex was calculated according to Elias (13).

RESULTS.

Solubilization.

When particulate receptor preparations, preincubated with ^{125}I-pFSH were extracted with N.P-40, 90 % of the bound radioactivity was recovered in the solubilized fraction. Partition chromatography of the soluble fraction on Sepharose 6B shows two peaks ; the front peak, representing the hormone-receptor complex, accounted for 60 percents of the total

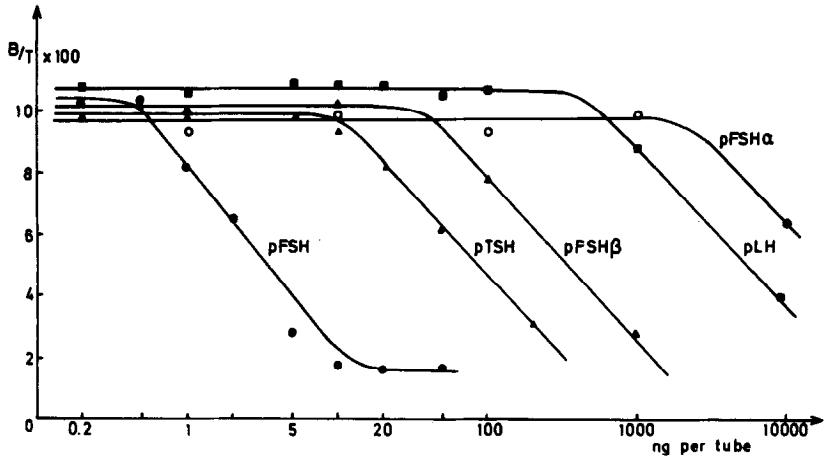


Figure 3. Inhibition of ^{125}I-pFSH binding by soluble receptors in the presence of increasing amounts of pFSH, pFSH α, pFSH β, pLH and pTSH.

radioactivity ; the second peak (40 % of the radioactivity) corresponded to the elution volume of ^{125}I-pFSH.

Eighty percent of the prelabeled receptor complex were precipitated by 1.2 M ammonium sulfate as final concentration. In the same conditions, no more than 3 % of free ^{125}I-pFSH were precipitated. Polyethyleneglycol between 8,9 % and 10,7 % (W/V) final concentrations precipitated 60 % of the soluble radioactivity ; however this procedure could not be used to separate receptor bound and free ^{125}I-pFSH since 25 % of free ^{125}I-pFSH precipitated at these concentrations.

Free FSH receptors were also solubilized by N.P-40. The number of free binding sites in each fraction was measured by Scatchard analysis of equilibrium binding. It appeared that 20 % of the total binding sites initially present in the particulate preparation were recovered in the supernatant of the 300,000 g centrifugation.

No more than 10 % of the total receptors were present in the pellet of the 27,000 and 300,000 x g fractions ; this indicates a substantial loss of free binding sites during the extraction procedure suggesting that the receptor in its free form is relatively labile. Protection of binding activity, during the extraction procedure and incubation, could not be achieved in the presence of 100 IKU/ml of trasylol, 0.02 M pMSF (para-methylsulfonylfluoride), 0.005 M EDTA, Lima and Soybean Trypsin inhibitors

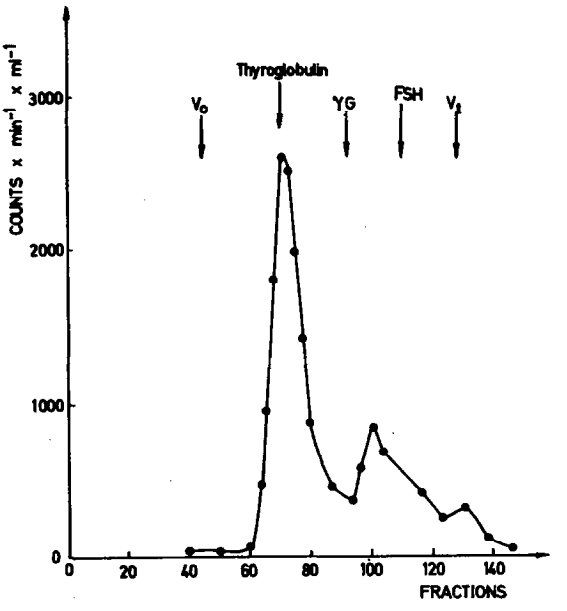


Figure 4. Gel filtration of ^{125}I-pFSH receptor complex on Sepharose 6B (column 1 x 90 cm) equilibrated in 0.05 M Tris-HCl buffer containing 0.1 % Nonidet P-40 and 0.2 % bovine serum albumin. Fraction size 0.7 ml ; flow rate : 12 ml/hour. The arrows indicate the elution volumes of marker proteins.

(0.2 mg/ml). Treatment of the soluble receptor preparation with trypsin (1 mg/ml) reduced the ^{125}I-pFSH binding of about 25 %, while exposure to neuraminidase (0.03 UI/ml), phospholipase A (0.5 U/ml) and phospholipase C (1 U/ml) did not significantly affect the binding.

Equally good results in solubilizing the FSH testis receptors were obtained with Triton X-100 and BRIJ at the same concentration while sodium deoxycholate, lithium diiodosalicylate, sodium dodecylsulfate and digitonine were found much less efficient.

Binding studies.

As shown in fig. 1 the binding of ^{125}I-pFSH to solubilized FSH receptors was a time dependent process. The hormone-receptor complex concentrations at steady state were lower at 34° and 24° than at 4°C suggesting the presence of a temperature dependent reactant inactivation process. Scatchard analysis of binding inhibition curves obtained at equilibrium (fig. 2) was linear, indicating a single order of

TABLE 1.

Physical parameters of the ^{125}I-pFSH receptor complex and free ^{125}I-pFSH derived from gel filtration on Sepharose 6B and Sephadex G-200 and density gradient centrifugation.

Parameter	Hormone-receptor complex	^{125}I-pFSH
Partition coefficient (Sephadex G-200)	0.14	0.58
Partition coefficient (Sepharose 6B)	0.34	0.70
Sedimentation coefficient (s_{20W})	7.9	2.9
Stokes radius (r)	58 Å	24 Å
Molecular weight[a] (M)	244,000	36,000

[a] The molecular weights (M) were calculated from the equation

$$M = \frac{6 \pi \eta r N s}{1 - \bar{\nu}}$$

The solvent density (ρ) was measured to be 1,002. The relative viscosity η_r of the solvent containing 0.1 % N.P-40 was determined in an Oswald viscosimeter to be 1,055. The viscosity (η) calculated from $\eta = \eta_r \times \eta_o$ was 0,0106 poise.
The densities of the hormone receptor complex and ^{125}I-pFSH measured in isopycnic density gradient centrifugation in cesium chloride were 1,294 and 1,301 respectively.
The specific volumes ($\bar{\nu}$) derived from these values were 0,772 and 0,768 respectively.

independent binding sites with high affinity (Ka = 4.7 x 10^{10} M^{-1}). This correlates well with the Ka (2 x 10^{10} M^{-1}) determined for the particulate binding sites (8). Fig. 3 demonstrates the specificity of the soluble FSH receptor. Indeed, LH, TSH, FSH α and FSH β did not inhibit FSH

binding, if one except the effect due to their respective contamination by native FSH as measured by radioimmynoassays (9,10,11).

Physical characteristics.

Chromatography of the soluble FSH receptor complex on Sepharose 6B (fig. 4), Sephadex G-200, ultracentrifugation in sucrose density gradient in the presence of marker proteins and isopycnic density gradients yielded informations concerning the molecular size and the hydrodynamics properties of the hormone-receptor complex. These results are summarized in table I. They are consistent with the parameters previously determined for the soluble receptor of HCG (5).

CONCLUSIONS.

In this work we have solubilized FSH receptors from porcine testis by the use of N.P-40. With respect to affinity and specificity, the FSH soluble binding sites possessed the same characteristics than the particulate receptors (8). A precise determination of the binding properties of the FSH soluble receptors was made possible by the use of tracer FSH with high binding activity (8) and by the development of a suitable procedure to separate receptor bound and free ^{125}I-pFSH. Indeed, ammonium sulfate precipitated selectively the hormone-receptor complex with minimal precipitation of free ^{125}I-pFSH. Unsuccessful attempts to solubilize FSH receptors with various detergents were reported by Bhalla and Reichert (14). This may be attributed to the rather low binding activity of chloramin T labeled ^{125}I-hFSH on rat testis receptors or to unadequate use of polyethyleneglycol to separate bound and free hormones.

Soluble binding factors for gonadotropins have been extracted by ethanol from rat testis (15). In contrast to the soluble FSH binding sites described in the present study, the factors extracted by ethanol have not yet been proven to be strickly specific for FSH.

The N.P-40 solubilized receptor for FSH was found by gel filtration and density gradient centrifugation to be comparable in shape and size to other detergent solubilized peptide-hormone receptors (5,16).

In conclusion, the procedures presented in this paper appear to be a useful preliminary step for the purification of FSH testis receptors.

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Note added in proof:

While our paper was submitted for publication, Abou Issa and Reichert published their data on the solubilization of FSH receptors from calf testis (J. Biol. Chem. 252, 4166, 1977).

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