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Gonadotropic hormone (GtH) receptors in the testis of the trout *Salmo gairdneri*: *in vitro* studies

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Keywords: trout, testis, membrane fraction, GtH, receptors, hormone and tissue specificity, affinity constant, androgen secretion, stimulation

Abstract

A particulate fraction obtained from trout testis at the time of spermiation shows saturable binding sites for ^{125}I -labeled salmon gonadotropin (^{125}I -GtH). Non-gonadal tissues (liver, muscle and spleen) did not demonstrate specific ^{125}I -GtH binding. The tracer's specific activity was determined by the self-displacement method (18 to 30 $\mu\text{Ci}/\mu\text{g}$). Maximal specific binding ability of ^{125}I -GtH varied from 20 to 30% of the labelled ligand added, depending on the hormone preparation. Specific binding of ^{125}I -GtH to 20 mg of the testis membrane varied from 40 to 85% of the total binding depending on the method of membrane preparation, and was competitively inhibited by concentrations of unlabelled GtH ranging from ca 1 to 1000 ng/ml of incubate. Gonadotropin of mammalian origin, ovine TSH or salmon prolactin competed only weakly, or not at all, for testicular gonadotropin binding sites (relative potencies s-GtH $\gg$ FSH = hCG $>$ s-PRL $>$ bTSH). Scatchard analysis of equilibrium binding studies shows that saturable gonadotropin binding was due to a class of high affinity binding sites (sites I $K_a \cong 3 \times 10^{10} \text{ M}^{-1}$) and possibly to a second class of lower affinity binding sites (sites II $K_a = 5 \text{ to } 14 \times 10^8 \text{ M}^{-1}$). The binding capacity of sites I, as measured in enriched membrane preparations, was $45 \pm 18 \text{ fmoles/g}$ of testis during the period of spermiation. The concentration of GtH required to obtain half maximal displacement of ^{125}I -GtH in the binding studies was of the same order of magnitude as the apparent ED₅₀ for GtH stimulation of 11-Cetotestosterone (11KT) secretion by trout testes *in vitro*. Mammalian LH and FSH were 100 to 1000 folds less potent than salmon GtH to increase 11 KT secretion.

Introduction

Variations in circulating gonadotropin (GtH) concentrations influence the gonadal functions in fish, but cannot always explain the various aspects of testicular activity. Other circulating factors and/or local regulations could modulate the response to GtH. One level for intragonadal regulation could be changes in the number, affinity, or even distribution of gonadotropin receptors in the testis.

Binding of iodinated human chorionic gonado-

tropin (hCG) to trout testis has been partially characterized (Schlaghecke 1983) and has been found to be inhibited by a trout pituitary glycoprotein (Schulz and Schlaghecke 1984). However, little information is available on the properties of fish gonadotropin binding to fish gonads. Specific binding sites have been demonstrated in immature chum salmon ovary (Van Der Kraak 1983) and in goby testes (Aida and Ishii 1981) and have been preliminarily described in isolated granulosa cells from Amago salmon ovary (Salmon *et al.* 1984). Carp

GtH binding to eel ovaries has been recently described (Salmon *et al.* 1987).

The aim of this study was to demonstrate and characterize, *in vitro*, the specific binding of purified maturational salmon GtH to a mature trout testis particulate fraction. The paper present here reports on the association and dissociation of iodinated GtH of a known specific activity with testicular binding sites, the tissue and hormonal specificity of the hormone-receptor complex formation, the determination of affinity constants and capacities of the testicular binding sites by saturation and competition experiments at equilibrium, and on the ability of various hypophyseal hormones to bind to GtH receptors and to activate steroid production by the trout testis *in vitro*.

Materials and methods

Experimental animals

Two to 3 year old spermiating male rainbow trout (*Salmo gairdneri*) (gonadosomatic index = 3–6%) reared at Gournay (Oise) fish farm, and acclimatized for 1 to 3 weeks in the recycling water unit of our laboratory (12°C), were used.

Hormones

GtH was purified from salmon (*Oncorhynchus tshawytscha*) pituitary glands as previously described (Breton *et al.* 1986). Ovine FSH (o-FSH – S 1) was obtained from NIH, Bethesda, MD, hCG Organon (Pregnyl) and bovine TSH (TS 10) from Sigma. Salmon prolactin was a gift from Dr. Prunet (INRA), Rennes, France.

Preparation of receptor material

The entire procedure was carried out on ice, using chilled buffers. Testes were minced, then homogenized in Tris 0.025 M, MgCl₂ 10 mM, buffer (pH 7.5), generally containing 0.15 M sucrose and various inhibitors of proteolytic enzymes (PMSF 1

mM; soya bean trypsin inhibitor 100 µg/ml; paraaminobenzamidine 1 mM) (40 ml / 10 g of starting tissue weight). A polytron tissue grinder, with 2 successive 15 seconds bursts at high speed, was used. The homogenate was passed through a nylon filter (pore size 150 µm) and centrifuged (1000 × g, 10 min at 4°C). The supernatant was centrifuged again at 30000 × g for 30 min and the resulting pellet was resuspended in the assay buffer (Tris 0.025 M, MgCl₂ 10% mM, BSA 0.1%; pH 7.5) (TMB) generally at 500 mg of pellet / ml. This suspension was kept at –70°C until used and was diluted to the desired concentration just prior to the binding assay.

Preparation of ¹²⁵I-GtH

Five µg of salmon GtH were iodinated by the lactoperoxidase method (Thorell and Johansson 1971) as modified by Hirano *et al.* (1985). Lactoperoxidase was purchased from Sigma and Na ¹²⁵I (IMS 30) from Amersham, Buckinghamshire U.K. Separation of radiolabelled GtH from free ¹²⁵I was carried out by chromatography on ACA 54 column (1 × 20 cm). The maximum binding activity of the labelled hormone was measured by incubation of 15,000 cpm of ¹²⁵I-GtH with an excess of receptor sites (30,000 × g fraction of the testis homogenate) and expressed as % of total added hormone. The values obtained show that only 20 to 30% of the hormone had the ability to bind to the receptor, depending on the batch of unlabelled hormone used to prepare the tracer (2 different batches were used). The specific radioactivity of the different batches of ¹²⁵I-GtH (SA) was determined by self-displacement in the radioligand-receptor assay (Catt *et al.* 1976) as shown in Fig. 1 and compared to SA = [radioactivity content / protein content] in the protein fraction eluted from the ACA 54 column. The values found using these two methods were similar during the first 5 days after iodination (18–30 µCi / µg). This, and the fact that tracer and unlabelled hormone had similar affinities for the receptor (see Fig. 5) show that iodination procedure had little effect on the hormone bindability. Therefore the loss of bindability of the hormone

Fig. 1. Determination of specific activity of ^{125}I -GtH by auto-competition. Specific binding, expressed as % of total ^{125}I -GtH, obtained by incubating 10 mg of membranes with increasing concentrations of ^{125}I -GtH: (—●—) (top abscissa, right ordinate), or with increasing concentrations of unlabelled GtH: (—○—) (lower abscissa, left ordinate).

must have occurred before iodination (during the various stages of purification, conservation at -20°C , freezing, thawing, etc.).

Binding assay procedure

Assays were performed in 5 ml plastic tubes containing 100 μl of assay buffer, 50 μl of ^{125}I -GtH, 100 μl of receptor preparation (30,000 $\times$ g pellet of the testicular homogenate) and 50 μl of assay buffer containing, if any required, unlabelled hormone as described in the results. The final reaction volume was 300 μl . After vortexing, the tubes were incubated at 16°C for 18 h, except in one experiment in which different times of incubation were tested. At the end of the incubation 1 ml of chilled assay buffer was added and the tubes were centrifuged at 2,000 $\times$ g for 15 minutes. The supernatant was discarded and the pellet washed a second time in the same way. The pellet associated radioactivity was counted in a Packard gamma counter (75% efficiency).

The nonspecific binding (NSB) was determined in the presence of an excess of unlabelled GtH (1000 ng/tube). The specifically bound (SB) radioactivity was calculated by subtracting the nonspe-

cifically bound (mean of triplicate determination) from the total bound radioactivity (mean of triplicate determination).

Calculation of the bound and free hormone concentrations

As previously shown, iodination by itself had no important effect on hormone bindability, and the specific activity (SA) determined previously was used to calculate the concentrations of specifically bound (SB) hormone as follows:

$$B = \text{SB cpm} / \text{SA}$$

The 'maximum binding' experiment showed that only a fraction ($y = 0,2$ or $0,3$) of the hormone had the capacity to bind to the receptor. Therefore, in equilibrium studies, the concentration of unbound hormone ($F = \text{free}$) able to bind to the receptor was calculated as follows:

$$F = \frac{(\text{Tcpm} - \text{NSB cpm}) \times y - \text{SBcpm}}{\text{SA}}$$

- Where — Tcpm = total added radioactivity
 — NSB: includes both active and inactive ^{125}I -GtH non-specifically bound.
 — SB, the radioactivity specifically bound to the receptors, is believed to represent only intact GtH.

In vitro steroid production

Testes from spermiating trouts were collected from freshly killed animals. The tissues were cut into 1 mm blocks, washed 3 times in culture medium, and incubated in 24 well-culture dishes (Nunc) for 20 hours at 14°C in air (20 blocks/well/ml medium). The incubation medium was TC-MEM (DIFCO; powder) supplemented with glutamine, sodium bicarbonate (0,3 mg/l) and buffered by Hepes NaOH 0,02 M at pH 7,5. Steroids were measured in the incubation media using the radioimmunoassay described by Fostier *et al.* (1982) ex-

Table 1. Comparison of different protocols for trout testis membrane preparation

Protocol		GtH binding ^a (fmol/g of testis)	Repartition in the fractions ^b	NSB ^c (% TB)
A. 1 centrifugation	12,000 × g 30 min	94	100% ?	45%
B. 1 centrifugation	30,000 × g 45 min	125	100%	30–40%
C. 2 centrifugations	1) 1,000 × g 10 min	50	64%	62%
	2) 30,000 × g 30 min	28	36%	15–30%
D. 3 centrifugations	1) 1,000 × g 15 min	78	77%	65%
	2) 20,000 × g 25 min	20	19%	25–30%
	3) 100,000 × g 90 min	4	4%	15%

^a Specific binding capacity was measured in 20 mg of the final pellets and reported per g of starting tissue; ^b the sum of the binding sites which were recovered in the different pellets of 1 protocol equals 100%; ^c non specific binding, in % of total binding, when using 20,000 cpm of total ¹²⁵I-GtH and 20 mg of pellet.

cept that the media were directly assayed without prior extraction.

Statistical analysis

Values for affinity constants (K_a), number of binding sites (R_0) and GtH concentrations inducing half maximum response (ED_{50}), given in the text, are the means of n experiments (done on n different fish) $\pm$ SEM.

Results

Preparation of receptors

Preliminary experiments were performed to compare the effect of various homogenization buffers on ¹²⁵I-GtH binding. The use of Tris 0.025 M, MgCl₂ 10 mM (pH 7.5) or PBS 10 mM (pH 7.5) gave similar results, while the addition of inhibitors of proteolytic enzymes to homogenization solutions proved to be necessary for the preservation of binding activity (data not shown).

Different centrifugation procedures commonly used for gonadotropin receptor preparation were compared. Homogenization and filtration steps were identical and protease inhibitors were added in all cases. Binding capacity of the various pellets

was measured using saturation experiments and is reported per g of starting tissue. The results summarized in Table 1, indicate that a one-step procedure allows a much higher recovery of the GtH binding sites initially present in the testis, since in a 2 or 3 step procedure a large percentage of receptors are lost during the first centrifugations (60 to 90% depending on the procedure). However, in crude homogenates the non specific binding is much higher than in partially purified membranes. For this reason, procedure C, in which 30 to 40% of total binding sites were recovered in the enriched membrane fraction and in which NSB was reasonable (15 to 30% of total binding, using 20,000 cpm of total ¹²⁵I-GtH) was selected for the remainder of the study.

¹²⁵I-GtH binding to particulate fraction of trout testis

Testes, liver, muscle and spleen were collected, and particulate fractions of these tissues prepared as described for testes (see above) and tested at different dilutions for ¹²⁵I-GtH specific binding (Fig. 2). Only testes displayed a significant specific binding; muscle showed a small degree of binding (0.5% of total added ¹²⁵I-GtH), at high tissue concentration (25 mg/tube).

When a constant concentration of tracer was in-

Fig. 2. Specific binding of ^{125}I -GtH to different trout tissues. One hundred μl aliquots of tissue homogenates at various dilutions were incubated as described in Materials and Methods, with 24,000 cpm of ^{125}I -GtH in the absence (TB) or presence (NSB) of $1\text{ }\mu\text{g}$ of unlabelled GtH. The specific binding (TB-NSB) is expressed in % of total added tracer.

cubated with increasing amounts of testis preparation, specific binding increased as a function of tissue concentration within the range 3.1–25 mg, after which binding tended to reach a plateau.

Association and dissociation studies

Effect of incubation time on the receptor hormone complex formation was studied at 16°C and pH 7.5. In these conditions apparent equilibrium was reached after 16 to 20 h of incubation (Fig. 3), after which specific binding was stable until at least 24 h of incubation. At 4°C the binding reaction was slower, but a similar maximum binding was reached at equilibrium.

Dissociation of GtH from its binding sites was studied after preincubation of membrane aliquots with ^{125}I -GtH. At the end of the preincubation the membranes were washed twice with ice-cold TMB to eliminate the unbound hormone, then resuspended in 2 ml of TMB. The remaining specific binding was determined at successive time intervals (Fig. 4). Dissociation was rapid during an early period, 25% of the complex being dissociated after 6h. Between six and 24 h dissociation was very slow and only 33% of the originally bound hormone

Fig. 3. Binding of ^{125}I -GtH to trout testis membranes as a function of incubation time at 4°C (—○—) and 16°C (—●—). Values are expressed as percentage of total added ^{125}I -GtH ($T_c = 15,000$ cpm) specifically bound to the testis homogenate (10 mg / tube).

Fig. 4. Time course of the dissociation of ^{125}I -GtH from testis receptor sites at 16°C , after preincubation of 10 mg homogenate with 20,000 cpm ^{125}I -GtH for 16 h. See text for details.

was dissociated after 24 h.

Saturation and competition experiments

When a constant amount of testis preparation was incubated with increasing concentrations of ^{125}I -GtH, NSB behaved linearly and was not saturable, while SB tended to be saturated at high tracer concentrations (Fig. 5A). When the incubation was made with a fixed concentration of labelled hormone, specific binding of ^{125}I -GtH could be competitively inhibited by increasing concentrations of unlabelled GtH (Fig. 6). Inhibition was significant with as little as 0.4 ng of GtH / ml ($p = 0.05$), while

Fig. 5. One representative experiment showing specific binding of GtH to trout testicular membranes as a function of hormone concentration. A: with increasing amounts of $^{125}\text{I-GtH}$, B: with increasing amounts of unlabelled GtH and constant amount of $^{125}\text{I-GtH}$ (20,000 cpm). C: determination of affinity constant (K_a) and number of GtH receptor sites (R_0). Scatchard plot of data obtained from saturation (—●—) and competition (—○—) experiments. The nonlinear Scatchard plot from competition data was obtained with a wide range of GtH concentrations (0.39 – 1,000 ng / tube) competing with a fixed amount of $^{125}\text{I-GtH}$ (20,000 cpm / tube, SA = 24 $\mu\text{Ci} / \mu\text{g}$) to bind to trout testis homogenate (10 mg / tube).

100 ng / ml inhibited about 90% of $^{125}\text{I-GtH}$ binding. The concentration of cold GtH required to obtain half maximum displacement of $^{125}\text{I-GtH}$ was 9.9 ± 2.4 ng / ml ($n = 5$).

The mammalian gonadotropins, oFSH and hCG, partially inhibited $^{125}\text{I-GtH}$ binding, but a 25 to 35% inhibition necessitated hormone concentrations 500 to 10,000 fold higher than when using salmon GtH (Fig. 6). One μg of salmon prolactin and 1 μg of bTSH could induce a 15% inhibition of SB but these hormones were ineffective at lower concentrations (Fig. 6).

Equilibrium affinity constants and binding site concentrations

Receptor binding data were analysed using the Scatchard method, in which the ratio of bound to free hormone is plotted against the specifically bound hormone concentration. If hormone-receptor interaction is a reversible bimolecular reaction, the plot results in a straight-line relationship with a slope of K_a , and the intercept of this line with the horizontal axis equals the total concentration of binding sites (R_0). In the hormonal concentration range lower than 30 ng / tube, both saturation and competition studies gave similar results (Fig. 5), providing evidence for a population of binding sites (sites I) with a high affinity for GtH (saturation studies: $K_a = 3.30 \pm 1.15 \times 10^{10} \text{ M}^{-1}$ ($n = 7$); competitions studies: $K_a = 2.42 \pm 0.73 \times 10^{10} \text{ M}^{-1}$ ($n = 5$)), and a limited capacity ($R_0 45 \pm 18$ fmoles / g of starting tissue; $n = 6$).

However, the use of higher concentrations of unlabelled hormone in the competition studies gives a non-linear result (Fig. 5c), which suggests that a second type of binding site (site II) exists, with a higher capacity (R_0 : 100 to 300 fmoles / g of testis) and a lower affinity ($K_a = 9 \pm 3 \times 10^8 \text{ M}^{-1}$ ($n = 4$)).

GtH stimulation of steroidogenesis in vitro

In Fig. 7, the left curve represents one of the four experiments in which GtH effect on 11 KT production by spermiating trout testes was tested *in vitro*. In these experiments salmonid GtH induced a dose-dependant stimulation of 11 KT production; the lowest efficient GtH concentration ranged from 1.56 to 6.25 ng / ml and maximum stimulation was reached between 50 and 200 ng / ml. The mean concentration of GtH inducing 50% of the maximum response (ED_{50}) was 9.3 ± 5.4 ng / ml ($n = 4$).

The mammalian gonadotropins, o-FSH and o-LH, also stimulated 11 KT production, but at concentrations 100 to 1000 folds higher than when using s-GtH. Human chorionic gonadotropin (hCG), up to 12 μg / ml (100 UI / ml) had no significant effect on 11 KT production.

Fig. 6. Competitive inhibition curves for specific binding of ^{125}I -GtH to trout testicular receptors by increasing concentration of native salmon GtH or various hormone preparations. Binding is expressed as % of ^{125}I -GtH specific binding in the absence of competitor.

Fig. 7. Effect of increasing concentrations of sGtH and mammalian gonadotropins on steroid production by incubated trout testicular tissue *in vitro*. 11 Keto-testosterone (11 KT) was assayed in the incubation medium; values are expressed as ng of steroid produced / mg of total testicular proteins. See text for details.

Discussion

In the present work, specific binding of GtH to the trout testis during spermiation was studied *in vitro*. The binding was saturable, indicating a finite number of binding sites, and presented a high affinity. Hormone and target organ specificity were also demonstrated. All these properties are characteristic of hormonal receptors.

The affinity constant found for the type I sites is two orders of magnitude higher than the K_a previously described for s-GtH binding to goby testis (4

$\times 10^8 \text{ M}^{-1}$, Aida and Ishii (1981)) and one order of magnitude higher than the K_a found for hCG binding to trout testis ($2, 7 \times 10^9 \text{ M}^{-1}$, Schlaghecke (1983)). We also found that oFSH and hCG could bind to trout testis, but with a much lower affinity as shown by the high concentrations of these mammalian hormones necessary to partially inhibit ^{125}I -GtH binding. The small effect obtained with b-TSH and s-prolactine would necessitate further experiments to know whether this inhibition is competitive.

From the K_a values obtained in this study and from the GtH circulating concentrations (0.1 to 0.5 nM) we calculated that 71% to 92% of sites I and 8 to 38% of sites II would be occupied at this stage of testicular development. However such a calculation is susceptible to error since we do not know the biological activity of GtH in the plasma (measured by radioimmunoassay). Furthermore K_a values were determined in conditions (pH, temperature, ionic concentrations) which allow optimal binding *in vitro* but which might be different from intratesticular conditions at the time of spermiation.

To our point of view, the functional significance of GtH receptors is more clearly established by showing a parallel increase of specific gonadotropin binding at equilibrium and of the steroidogenic response at various levels of added hormone. The ED 50 for the stimulated 11 KT production was in the same order of magnitude as the concentration necessary to obtain a half maximum inhibition of the ^{125}I -GtH binding in the competition studies.

Conversely, poor competitors like o-FSH need to be added at supraphysiological concentrations to affect 11 KT production *in vitro*. Human CG recognized GtH binding sites, as previously shown by Schlaghecke (1983). However in this experiment hCG had no further effect on androgen secretion. This is consistent with previous studies showing that hCG is 25,000 fold less active than homologous GtH on androgen formation by minced carp testis (Yu and Lin 1986).

We have shown that competition experiments with unlabelled GtH give rise to two different slopes in the Scatchard analysis. Non-linearity of the Scatchard plot is generally attributed to heterogeneity of the receptor sites, with the presence of

two 'orders' of affinity. The biphasic dissociation kinetics found in this study might also be taken to imply a heterogeneity of receptor sites (Rodbard 1973). Binding tests performed on fresh tissue, or on more purified membrane fractions also revealed two classes of binding sites (data not shown). The presence of two classes of sites is also suggested in the trout ovary (Breton *et al.* 1986). In the testis, two classes of GtH binding sites could correspond to two biological effects of the hormone in one cell type, or it could indicate the existence of two types of receptors localized in two different populations of target cells. Such an hypothesis, previously proposed by Fontaine (1980), would be particularly attractive in the male fish model, where GtH can stimulate androgen production and can control all the stages of spermatogenesis (see Billard *et al.* 1982) while in mammals two hormones, FSH and LH regulate the activity of the two testicular compartments (respectively seminiferous tubules and interstitial tissue).

However, the existence of two classes of receptors needs to be seriously tested. Nonlinear Scatchard may be induced by the presence of 'negative cooperativity', or contamination of the hormone with damaged molecules presenting lower affinity (for review see Rodbard 1973). Similarly, the second slow dissociation phase could be related to an irreversible binding, as has been shown to precede internalization of LH in intact cells and even to occur in acellular incubation systems (Catt *et al.* 1980; Ketelslegers *et al.* 1975). Therefore, the interpretation of our results as indicative of receptor heterogeneity must be regarded as provisory.

Variations were observed in K_a values and number of sites measured in different experiments, using different fish. This could be due to inter-assay variation (not determined in this series of experimentations), but it could also be a consequence of differences in the precise developmental stage of the spermiating testicules used. We have now started to investigate eventual changes in the affinity and number of GtH receptors (at least on sites I) during the reproductive cycle in relation with gonadosomatic index, testicular histology and plasmatic GtH concentrations.

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