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Cultured Pituitary Cell GtH Response to GnRH at Different Stages of Rainbow Trout Spermatogenesis and Influence of Steroid Hormones¹

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Using primary cultures of whole dispersed pituitary cells collected from rainbow trout at different stages of spermatogenesis, basal and GnRH-induced GtH release and cell GtH content were studied in control and steroid-pretreated cultures. Steroid pretreatments were performed for 3 days with 11-ketotestosterone (11KT) and 17 α -hydroxy,20 β -dihydroprogesterone (17 α 20 β P) at levels corresponding to those circulating at the time of spermiation (50 and 20 ng/ml, respectively). In control cultures, basal GtH release and cell GtH content increased with the stage of spermatogenesis in a characteristic pattern as predicted from *in vivo* results concerning plasma and pituitary GtH contents. The pituitary response to salmon GnRH (sGnRH) also varied as indicated by the decrease in the minimal effective dose of GnRH able to induce a significant GtH release with the advancement of spermatogenesis: 10⁻⁷ M at the spermatocyte stage, 10⁻⁹ M at prespermiation and spermiation. Steroid pretreatments were shown to have a direct effect on pituitary gonadotrophs and particularly on pituitary response to sGnRH, depending on the stage at which they are applied. At the beginning of spermatogenesis both of them induced an increase in GtH release and at prespermiation they have a slight negative effect, significant only with 17 α 20 β P. At spermiation they have no effect except for 17 α 20 β P which increased the response to 10⁻⁸ M of sGnRH. Results are discussed in relation to hormonal changes (gonadotropin and steroid) observed by different authors during the sexual cycle. © 1990 Academic Press, Inc.

Previously, we have studied *in vivo* the pituitary response to LHRH at different stages of spermatogenesis in intact (Weil *et al.*, 1978) and castrated (Weil, 1981) rainbow trout. The response of intact fish to LHRH, characterized by the percentage of fish having a GnRH-induced gonadotropin (GtH) release, was absent at the beginning of spermatogenesis and increased during gonad maturation. In castrated fish, plasma GtH increased in comparison to controls as previously reported (Billard *et al.*, 1976) whereas no change in pituitary responsiveness to LHRH was observed at prespermi-

ation and spermiation but it tended to increase at the beginning of spermatogenesis (Weil, 1981). We have now subjected primary pituitary cell cultures to GnRH action at various stages of spermatogenesis. In order to study the direct role of steroid hormones on pituitary, basal and GnRH-induced GtH release have been recorded after *in vitro* pretreatment with 11-ketotestosterone (11KT) and 17 α -hydroxy, 20 β -dihydroprogesterone (17 α 20 β P), two major steroids at the time of spermiation (Campbell *et al.*, 1980).

MATERIALS AND METHODS

Animals

Work was begun in June 1985 with males hatched from a fertilization performed in January 1983 with a winter-spawning strain reared at the Gournay/Aronde

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fish farm. Prior to use, fish were kept some months in the laboratory in a recirculated water unit at 12°–13°.

Experiments were performed with fish at different stages of spermatogenesis. Spermiation was detected by stripping the fish; other stages were determined by macroscopic observation of the gonads using the criteria described by Billard and Escaffre (1975). The different stages studied were beginning of spermatogenesis (June 1985) and meiosis (July 1985), prespermiation (October 1985), and spermiation (January 1986 and March 1986).

Pituitary Response to Salmon GnRH (sGnRH) in Control and Steroid-Pretreated Cultures

Pituitary collection. At each defined stages of spermatogenesis, 10 to 30 animals were anesthetized with phenoxy-ethanol before pituitary removal, as described for females (Weil and Marcuzzi, 1990).

Cell cultures. Cultures were performed as described for such a study in females (Weil and Marcuzzi, 1990), using the technique previously set up for this species (Weil *et al.*, 1986).

Control and steroid pretreatments. As soon as the cells were plated into the wells, the steroid and control pretreatments were applied for 3 days during cell attachment to the plastic according to Weil and Marcuzzi (1990).

The following steroids were tested:

—11-ketotestosterone at a dose of 50 ng/ml, which is the mean plasma level at the time of spermiation (Foster *et al.*, 1982).

—17 α -hydroxy,20 β -dihydroprogesterone at a dose of 20 ng/ml, which is the mean plasma level at the time of spermiation (Scott and Baynes, 1982).

Incubation with GnRH. On the third day of culture, control and steroid-pretreated wells were subjected to increasing doses of GnRH (0 to 10⁻⁶ M) during a 24-hr incubation period, as previously described (Weil and Marcuzzi, 1990).

Data Analysis

Parameters recorded. At each defined stage of spermatogenesis the following parameters were recorded in control and steroid-pretreated cultures:

—basal GtH release: GtH levels in wells without GnRH stimulation (dose 0 of GnRH).

—GnRH-induced GtH release for each tested dose.

—pituitary GtH content in control and GnRH-stimulated wells.

These parameters were estimated using a radioimmunoassay as previously described for assaying plasma and pituitary trout GtH content (Breton *et al.*, 1983) and previously validated for GtH measurement

in cells and culture medium (Weil *et al.*, 1986). They were expressed in nanograms of GtH per well.

Statistical analysis. Means were compared by an analysis of variance or a nonparametric test (Mann-Whitney *U* test). At each stage of spermatogenesis, the minimal effective dose of GnRH to induce GtH release was determined by comparing the mean GtH release using the Newman-Keuls test.

RESULTS

Response to GnRH at Different Stages of Spermatogenesis

At the beginning of spermatogenesis (spermatogonia A and B, GSI: 0.54 $\pm$ 0.03%), basal GtH release is low 2.00 $\pm$ 0.86 ng/well as well as GnRH-induced GtH release; no significant increase was observed with the different doses of sGnRH, when compared with the basal level (Fig. 1).

When active spermatogenesis begins (presence of spermatocytes in all the fish and spermatids in some of them, GSI: 1.85 $\pm$ 0.23%), basal GtH release is still low (2.84 $\pm$ 1.09 ng/well). In contrast, gonadotrophs present in the culture are responsive to GnRH. Doses ranging from 10⁻⁹ to 10⁻⁷ M induced a twofold increase compared to the basal level, at the limit of significance,

FIG. 1. Response to various doses of sGnRH of pituitary cell cultures from males at the beginning of spermatogenesis during a 24-hr incubation period, following a 3-day pretreatment in control medium (●) or in medium supplemented with 11KT, 50 ng/ml (○), or 17 α 20 β P, 20 ng/ml (■). For this and subsequent figures wells were initially plated with 6.2×10^4 cells. Values are $\bar{x} \pm$ SEM ($n = 5$).

whereas a significant 13.6 times increase ($P < 0.001$) was observed at the 10^{-6} M dose (Fig. 2).

At prespermiation (full spermatogenesis without spermiation, GSI: $4.67 \pm 0.39\%$), basal GtH release was 5.72 ± 0.83 ng/well and the cells significantly ($P < 0.025$) responded to 10^{-9} M of sGnRH and the maximal increase for 10^{-7} M was 3.4 times the basal level (Fig. 3).

At spermiation (emission of spermatozoa, GSI: $3.29 \pm 0.24\%$), basal GtH release was 10.51 ± 1.77 ng/well. Concerning responsiveness to sGnRH the minimal effective dose was 10^{-9} M and the maximal increase for 10^{-7} M of sGnRH was five times higher than the basal level, $P < 0.001$, (Fig. 4).

Cell GtH content increased with the advancement of spermatogenesis, the highest values being recorded at the time of spermiation (Table 1).

Influence of Steroid Pretreatments on Pituitary Response to sGnRH

At the beginning of spermatogenesis

FIG. 2. Response of pituitary cultures from males at the meiosis stage to various doses of sGnRH during a 24-hr incubation period, following a 3-day pretreatment in control medium (●) or in medium supplemented with 11KT, 50 ng/ml (○), or 17α20βP, 20 ng/ml (■). Values are $\bar{x} \pm \text{SEM}$ ($n = 5$).

FIG. 3. Response of pituitary cell cultures from prespermiating males to various doses of sGnRH during a 24-hr incubation period, following a 3-day pretreatment in control medium (●) or in medium supplemented with 11KT, 50 ng/ml (○), or 17α20βP, 20 ng/ml (■). Values are $\bar{x} \pm \text{SEM}$ ($n = 5$).

(spermatogonia A and B), a pretreatment of the pituitary cells with 11KT and 17α20βP induced a significant increase ($P < 0.05$ and $P < 0.01$, respectively) in GnRH-induced GtH release when compared with control wells (Fig. 1), while cell GtH was unchanged (Table 1). These same pretreat-

FIG. 4. Response of pituitary cultures from spermiating males to various doses of sGnRH during a 24-hr incubation period, following a 3-day pretreatment in control medium (●) or in medium supplemented with 11KT, 50 ng/ml (○), or 17α20βP, 20 ng/ml (■). Values are $\bar{x} \pm \text{SEM}$ ($n = 5$).

TABLE 1
EFFECT OF STEROID PRETREATMENTS ON GtH
CONTENT OF PITUITARY CELL CULTURES SET UP
WITH MALES AT DIFFERENT STAGES
OF SPERMATOGENESIS

Stages	Steroid Pretreatment	Cell GtH content ^c
Beginning of spermatogenesis	0	66.51 ± 15.15
	11KT ^a	79.23 ± 16.49 ^d
	17α20βP ^b	79.69 ± 17.03 ^d
Spermatocytes spermatids	0	95.64 ± 7.24
	11KT	106.40 ± 12.14 ^d
	17α20βP	97.11 ± 5.85 ^d
Prespermiation	0	174.15 ± 3.87
	11KT	145.53 ± 9.07 ^{e,*}
	17α20βP	154.13 ± 13.85 ^d
Spermiation	0	550.62 ± 51.07
	11KT	574.02 ± 48.47 ^d
	17α20βP	374.22 ± 26.59 ^{e,**}

^a 11 Ketotestosterone, 50 ng/ml.

^b 17α-Hydroxy-20β-dihydroprogesterone, 20 ng/ml.

^c Values are $\bar{x} \pm \text{SEM}$ and expressed as nanograms per well.

^d Values not significantly different from control.

^e Value significantly different from control (* $P < 0.008$; ** $P < 0.16$).

ments, applied to cultures set up with pituitary collected from males with gonads containing spermatocytes and spermatids, were without any effect on GnRH-induced GtH release (Fig. 2) and on cell GtH content (Table 1). At prespermiation, a decrease is observed in GnRH-induced GtH release following steroid pretreatment but it is significant ($P < 0.05$) only with 17α20βP (Fig. 3); on the other side 11KT induced a decrease ($P < 0.08$) in cell GtH content (Table 1). At spermiation, steroid pretreatment did not modify the response to sGnRH except 17α20βP which increased ($P < 0.05$) the response to 10^{-8} M (Fig. 4) while cell GtH content was significantly ($P < 0.016$) decreased when compared with controls (Table 1).

DISCUSSION

We have studied, throughout the male rainbow trout sexual cycle, the response to sGnRH as well as basal and cell GtH content of the gonadotrophs present in pituitary primary cell cultures. The results *in vitro* are roughly in accordance with those previously obtained *in vivo* (Weil *et al.*,

1978) with intact fish. *In vitro* basal GtH level may be compared to the circulating GtH level and cell GtH content to pituitary GtH level; both of these parameters increased with the maturation of the testis. The response, respectively, to LHRH *in vivo* (1 μg/kg, administered by intracardiac injection) and to sGnRH *in vitro* (0 to 10^{-6} M) varied throughout the sexual cycle. *In vivo*, the response was undetectable at the beginning of spermatogenesis when low plasma and pituitary GtH levels were measured. *In vitro*, as well, no significant response was observed for all the tested doses of sGnRH and basal GtH level was low. The response began to appear *in vivo* in some fish when spermatocytes and spermatids were observed in the gonad and plasma GtH levels began to increase. At this stage, a response to GnRH was observed *in vitro*, an important increase being recorded, with the highest dose tested (10^{-6} M). At the time of prespermiation and spermiation, some fish kept being responsive to LHRH *in vivo* with a kind of pulsatile secretion at the latter stage in both control and LHRH-treated fish. *In vitro*, at both stages the pituitary responded to a low dose of sGnRH (10^{-9} M) with a greater increase compared to the basal level at spermiation.

This variation in the response to GnRH could be due to changes in circulating steroid levels and in the present work, we have tested this hypothesis by studying the direct effect of 11KT and 17α20βP on cultured pituitary cells pretreated for 3 days with either steroid. Both the plasma 11KT level (Scott and Baynes, 1982; Fostier *et al.*, 1982, 1984; Baynes and Scott, 1985; Lou *et al.*, 1986; Scott and Sumpter, 1989) and the 17α20βP level increase at the time of spermiation in rainbow trout (Scott and Baynes, 1982; Baynes and Scott, 1985; Lou *et al.*, 1986; Scott and Sumpter, 1989). However, 11KT and 17α20βP increases are out of phase, the first one peaking while the second one is just starting. These two steroids can act directly by increasing the re-

sponse to sGnRH when applied to cultured pituitary cells collected from males at an early stage of development of the gonads, whereas basal levels and cell GtH content remained unchanged. The high pituitary response to GnRH, observed *in vitro* (the present work) and *in vivo* at prespermiation and spermiation, could be due in part to the circulating levels of 11KT and $17\alpha 20\beta$ P since a pretreatment with these steroids increases the GnRH-induced GtH release of cultures at the beginning of spermatogenesis. At prespermiation $17\alpha 20\beta$ P tends to have a negative effect on the response to sGnRH of cultured gonadotrophs already primed by circulating levels whereas the effect of 11KT is not significant. This weak decrease in the response to sGnRH, linked to the presence of $17\alpha 20\beta$ P and the negative effect of 11KT on cell GtH content, might explain in part the slight decline in plasma GtH observed at the onset of spermiation (Sanchez-Rodriguez *et al.*, 1978; Fostier *et al.*, 1982). At spermiation 11KT was without effect on the pituitary response to sGnRH while $17\alpha 20\beta$ P induced an increase in GtH released following the administration of 10^{-8} M sGnRH, cell GtH content being reduced. These two observations may explain the increase in plasma GtH observed at full spermiation observed after the initial decrease (Sanchez-Rodriguez *et al.*, 1978).

An inhibiting action of the gonads on the hypothalamus-hypophyseal axis in adult male rainbow trout is indicated by the increased plasma GtH following castration at various stages of spermatogenesis (Billard *et al.*, 1976; Billard, 1978; Weil, 1981). However, the heterogeneity observed between individuals made it difficult to demonstrate a relationship with a variation in pituitary sensitivity to LHRH (Weil, 1981). Furthermore, the negative influence of gonads on hypothalamus-hypophyseal axis was shown by recent data obtained on other species (winter flounder: Crim *et al.*, 1987; cat fish: De Leeuw *et al.*, 1987) by

indicating an increase in the GnRH binding capacity following castration. The GnRH binding results following castration suggest that steroid treatments should decrease the response to GnRH of cultured gonadotrophs by decreasing binding to the receptors. However, the techniques used to study the retroaction of the gonads, castration and steroid pretreatment of pituitary cultures, are quite different. The effect of castration on plasma GtH is the sum of multiple effects on the hypothalamus-hypophyseal axis and may include gonadal factors other than steroid ones since an inhibin-like factor is suspected in rainbow trout (Breton and Billard, 1980). Furthermore, following castration, circulating steroids are not completely abolished in rainbow trout as are total androgens (Schreck *et al.*, 1972; Weil, 1981) and 11KT in winter flounder (Crim *et al.*, 1987); $17\alpha 20\beta$ P has not been studied but it is known that it is produced by other tissues such as the head kidneys (Sangalang and Freeman, 1988). Recent work, performed on rats, showed that a purified bovine inhibin decreased the number of GnRH receptors (Wang *et al.*, 1988). In fish, the suppression of this factor by castration might suppress this inhibitory influence, inducing an increase in circulating GtH levels via an increase in GnRH receptor number.

We have demonstrated that 11KT and $17\alpha 20\beta$ P have a different effect on the pituitary response to sGnRH, depending on the stages at which they are applied. Further work is necessary to determine the respective roles of other steroid and nonsteroid (inhibin-like) factors on the pituitary response to GnRH and on GnRH binding as soon as such an assay is available for this species.

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