Use of Pituitary Cells in Primary Culture to Study the Regulation of Gonadotropin Hormone (GtH) Secretion in Rainbow Trout: Setting Up and Validating the System as Assessed by Its Responsiveness to Mammalian and Salmon Gonadotropin Releasing Hormone¹

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To study the regulation of gonadotropin secretion in rainbow trout *in vitro*, a method for preparing primary cultures of dispersed pituitary cells is described. Cells were dispersed by collagenase 0.1% in Hank's saline solution for 20 hr at 12° and a high yield of viable cells was obtained. Attempts to improve cell functioning were made by varying culture conditions (density of cells initially plated, age of the culture). Cell functioning was assessed by their ability to respond to increasing doses of mammalian and salmon GnRH. Pituitaries were collected from spermiating males whose pituitaries are known to be sensitive to mammalian GnRH *in vivo*. Using 96-well plates, optimal conditions for good biological activity, are initial plating with 6.2×10^4 cells, incubation with GnRH for 24 hr on the third day after plating. In these conditions mammalian analog and salmon GnRH induced an increase in GtH release for doses ranging from 10^{-9} to 10^{-6} M. The GtH released during the GnRH incubation period does not decrease the sensitivity of the system since addition of 20 ng of GtH at the beginning of incubation does not modify the response profile. © 1986 Academic Press, Inc.

In previous work we have studied the regulation of GtH secretion by mammalian gonadotropin releasing hormone (mGnRH) *in vivo* in carp and trout (Weil *et al.*, 1975, 1978). The use of *in vitro* systems for studying the regulation of gonadotropin hormone (GtH) secretion provides a number of advantages over *in vivo* experimentation. In such a system the pituitary is isolated from neural influences and the action of test substances can be easily studied.

The use of dispersed pituitary cells is more suitable than the use of whole organs or half organs because it requires less donor animals and provides homogeneous

replicates since the pituitaries are pooled. These dispersed cells can be used in monolayer culture (static model) or anchored to beads and used in a perfusion system (dynamic model). In the former case each well is used for a test substance whereas in the latter, cells are usually used for several tests after a recovery period. For this reason we preferred to use a primary culture in a static model. However, dispersion and culture conditions can affect cell functioning and therefore the optimization of these parameters can greatly increase the usefulness of this technique. In the present work, we decided to study a few such parameters. Cell functioning was assessed by the ability of gonadotrophs present in the primary cell culture to respond to mammalian GnRH analog (D-Ala⁶ LHRH) and salmon GnRH (sGnRH) whose structure

¹ The results were presented in part as a poster at the 7th Conference of the European Society for Comparative Physiology and Biochemistry, Barcelona, Spain. August 26-28, 1985 (Weil, 1985).

has been recently determined (Sherwood *et al.*, 1983). Trials were conducted on adult spermiating trout which are known to have high pituitary GtH levels and to be responsive to mGnRH *in vivo* (Weil *et al.*, 1978).

MATERIALS AND METHODS

Animals. All the studies were conducted on 2-year-old spermiating males or on those just beginning regression of a spring-spawning strain reared at the fish farm of Gournay (Oise).

Cell preparation. Pituitary glands were removed from 10 to 20 fish and placed in ice-cold Hank's balanced salt solution (GIBCO Laboratories), buffered with 20 mM Hepes (P-L Biochemicals, Inc.) and 9 mM sodium bicarbonate as indicated by Wolf (1982) and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml) (GIBCO Laboratories). The buffered saline solution had an osmotic pressure of 300 mOsm/kg and a pH of 7.5, characteristic values of rainbow trout blood plasma. Pooled glands were rinsed with the buffered saline solution and chopped into small pieces with a surgical blade. The tissue fragments were transferred for dispersion to a siliconized glass-stoppered flask filled with 20–30 ml of Hank's buffered solution containing 0.1% (wt/vol) collagenase (Boehringer Mannheim) and 3% (wt/vol) BSA (RIA grade—Sigma). The mixture was incubated at 12° for 20 hr and during this period dissociation was mechanically aided by aspirating the fragments three times in a siliconized Pasteur pipette. Cells were harvested by centrifugation at 200g for 10 min at 15° and washed twice with the culture medium. The culture medium consisted of RPMI (GIBCO Laboratories) containing 20 mM Hepes, 9 mM NaHCO₃ and had a pH of 7.5 and an osmotic pressure of 300 mOsm/kg. This culture medium was supplemented with 2% of a serum substitute, ultrosor G or SF (IBF) and with the same dosage of antibiotic–antimycotic as previously described. Cells were resuspended in 1 ml of culture medium. Cell counting and cell viability were determined by mixing 40 µl of the cell suspension with 10 µl of 0.1% trypan blue (BDH chemicals) in 0.01 M phosphate buffer (pH 7.3) and examining the cells in a Thoma hemocytometer. After counting, the cells were diluted to 2.5×10^5 cells or 1.25×10^5 cells/ml of medium and 250 µl of suspension (i.e., 6.2×10^4 cells or 3.1×10^4 cells) were plated in each well of 96-well plates (NUNC). The dishes were maintained at 18°.

Microscopical observation of the cells was performed each day after plating. After 2, 3, or 5 days of culture (preincubation), the cells were washed twice with 250 µl of ultrosor and antibiotic–antimycotic free culture medium and subsequently reincubated in 250 µl of this medium with or without increasing concen-

trations of synthetic GnRH. A synthetic analog of LHRH, des-Gly¹⁰ D-Ala⁶ LHRH ethylamide (Beckmann), as well as a synthetic salmon GnRH (Breton *et al.*, 1984) synthesized according to the sequence described by Sherwood *et al.* (1983) were used at different doses (10^{-12} to 10^{-6} M). At the end of the incubation period (at 18° for 6 to 24 hr) the media were collected, centrifuged, and stored at –20° for subsequent determination of GtH. The intracellular concentration of GtH was determined in some experiments. After the removal of the incubation media, culture dishes were washed twice with buffered saline solution and 200 µl of radioimmunoassay buffer containing 0.1% (vol/vol) of Triton X-100 (Serva) was added to each well for 3 to 4 hr at 18° until complete breaking up of the cells was achieved as assessed by microscopic observation. The cell lysate was stored at –20° until GtH measurement. Results were expressed as nanograms of GtH released or present in the cell extracts per number of cells initially plated for the specified time intervals.

The overload of the medium by GtH was made by adding 20 ng/well of a pure salmon GtH (Breton *et al.*, 1978).

GtH measurement. A radioimmunoassay (RIA) established for assaying plasma and pituitary GtH content was used as described by Breton *et al.* (1971b). This RIA used a pure salmon GtH as a standard against guinea pig anti-trout GtH. Assessment of this RIA for measuring GtH contained in the cells and in the culture medium was performed by comparing the parallelism of the usual standard curve and that of the standard curve containing 0.1% Triton or culture medium in a logit versus log dose representation.

Statistical analysis. The data were analyzed using an analysis of variance and comparisons between means were made using the Newman–Keuls test.

RESULTS

Dissociation and Culture Procedures

The exposure of pituitaries to collagenase at 12° for 20 hr produced a high yield of single cells but some clusters were also observed. Cell viability tested immediately after dissociation was high and the mean yield of live cells was $88.98 \pm 1.49\%$ for the three trials reported in the present work.

Microscopically, freshly dispersed cells had spherical shapes. After 24 hr in culture at 18° they still had this shape and had begun division. By Day 2, the cells had flattened, spread, and begun to be at-

tached. The entire area of the dish was not covered with cells when initial plating is done with 6.2×10^4 cells. On the other hand by Day 3 cells were firmly attached and the entire area was covered with cells and by Days 4 and 5 the wells had the same appearance.

By Day 3, when wells were initially plated with half the quantity of cells (3.1×10^4 cells) the density of spread cells was equivalent to that observed on Day 2 after an initial plating of 6.2×10^4 cells per dish.

Assessment of the Biological Activity of Pituitary Gonadotrophs Present in the Primary Culture

Cells (6.2×10^4) were initially plated in control and experimental dishes. The biological activity of the pituitary gonadotrophs was tested by their ability to respond to GnRH stimulation after 3 days of preincubation. At that time cells were firmly attached, had spread and had covered the entire dish area.

(1) *Time course of GtH release by control and 10^{-8} M D-Ala⁶ LHRH stimulated plates (Fig. 1).* GtH release was studied 6, 12, and 24 hr during the incubation period in control and experimental quadruplicates. GtH levels rose slowly in the media collected from control dishes whereas a marked increase was noticed in GnRH stimulated ones. However, GtH levels were highly different from control ones

FIG. 1. Time course of GtH release by control and 10^{-8} M D-Ala⁶ LHRH stimulated wells initially plated with 6.2×10^4 cells. Values are $\bar{x} \pm \text{SEM}$ ($n = 4$). $**P < 0.005$.

only at 24 hr ($P < 0.005$). As a consequence a period of 24 hr was used in subsequent incubations.

(2) *GtH release with various concentrations of D-Ala⁶ LHRH or sGnRH by cultured pituitary cells (Fig. 2).* Quadruplicates of monolayer cultures were submitted for 24 hr to increasing doses (0 to 10^{-6} M) of D-Ala⁶ LHRH and sGnRH, respectively. Doses ranging from 10^{-12} to 10^{-10} M of both types of GnRH did not significantly change GtH release in comparison with controls (dose 0) and mean GtH levels were 7.62 ± 0.39 ng/ 6.2×10^4 cells. On the other hand, doses ranging from 10^{-9} to 10^{-6} M induced a significant increase compared with controls and mean GtH levels were, respectively, 16.34 ± 0.96 ng/well with D-Ala⁶ LHRH and 16.69 ± 0.56 ng/well with sGnRH.

Influence of the Preincubation Duration and of Cell Density Initially Plated on the Biological Activity of the Gonadotrophs in Primary Cell Culture as Assessed by Their Response to Incubation with GnRH

During a 24-hr incubation period increasing doses of GnRH (0 to 10^{-6} M) were

FIG. 2. GtH release with various concentrations of D-Ala⁶ LHRH and sGnRH by 6.2×10^4 cells initially plated, during the 24-hr incubation period. Incubation was performed 3 days after plating. Values are $\bar{x} \pm \text{SEM}$ ($n = 4$).

tested on preaduplicate wells either 2, 3, and 5 days after an initial plating with 6.2×10^4 cells or 3 days after an initial plating with 3.1×10^4 cells. In each experimental condition the biological activity of the gonadotrophs was assessed by studying basal GtH release (GtH present in the dose 0 wells at the end of the incubation period), cell GtH content (measured at the term of the incubation period), and the responsiveness to various doses of sGnRH (GtH present in the GnRH stimulated wells at the end of the incubation period).

(1) *Basal GtH release and cell GtH content* (Table 1). As the different doses of sGnRH treatment did not influence cell GtH content ($P > 0.05$), this parameter is expressed by the mean of all the wells.

Similar basal GtH levels and cell GtH contents were observed 2 days after an initial plating with 6.2×10^4 cells and 3 days after an initial plating with 3.1×10^4 cells. These values were significantly lower than those noticed in wells initially plated with 6.2×10^4 cells and preincubated for 3 and 5 days. These observations are in agreement

with the microscopic observation of cell development in the dishes and related above.

(2) *Responsiveness to GnRH* (Fig. 3). Following an initial plating with 6.2×10^4 cells, only the highest tested dose of sGnRH (10^{-6} M) induced a significant GtH release compared to basal level when incubation is performed after 2 and 5 days of preincubation. On the other hand, a significant GtH release was observed for doses ranging from 10^{-8} to 10^{-6} M when incubation is performed after 3 days of preincubation. As mentioned above, increase in GtH release was gradual and began at a dose of 10^{-9} M. On Day 3 basal and sGnRH induced GtH release presented good homogeneity among replicates and the global coefficient of variation was 18%. On the other hand, 5 days after plating these two parameters presented heterogeneous values. The global coefficient of variation was 63% and it is the reason why, a significant increase in GtH release was noticed only for the highest tested dose (10^{-6} M).

Following an initial plating with a low

TABLE 1

INFLUENCE OF THE PREINCUBATION DURATION AND OF THE CELL DENSITY INITIALLY PLATED ON BASAL GtH RELEASE AND GtH CONTENT OF GONADOTROPHS PRESENT IN PRIMARY CELL CULTURE AND SUBMITTED TO A 24-HR INCUBATION PERIOD WITH sGnRH

	Number of cells initially plated	Days of preincubation		
		2	3	5
Basal GtH release ^{a,b}	6.2×10^4	$0.85 \pm 0.35^*$ (4)	7.72 ± 0.64 (4)	5.35 ± 1.86 (4)
	3.1×10^4		$0.99 \pm 0.29^*$ (4)	
Cells GtH content ^{a,c}	6.2×10^4	$84.51 \pm 4.61^{**}$ (24)	132.94 ± 4.12 (24)	132.26 ± 6.66 (24)
	3.1×10^4		$84.52 \pm 4.93^{**}$ (24)	

^a Values are $\bar{x} \pm \text{SEM}$ and expressed as nanograms per number of cells initially plated. () = number of replicates.

^b Corresponds to the response to the 0 dose of sGnRH (see the results).

^c Values are the mean of control and sGnRH treated wells since the different doses of sGnRH did not influence cell GtH content (see the results).

*** Values significantly different from wells initially plated with 6.2×10^4 cells and studied after 3 and 5 days of preincubation. * $P < 0.02$, ** $P < 0.001$.

density of cells (3.1×10^4 cells) a response to GnRH was observed on Day 3 only for the highest tested dose (10^{-6} M).

Influence of an Overload of GtH in the Media on Gonadotrophs Responsiveness to Varying Doses of sGnRH

Three days after plating, quadruplicate dishes containing 6.2×10^4 cells initially were submitted for a 24-hr incubation period to 20 ng of GtH plus increasing doses of sGnRH (0 – 10^{-6} M) or to sGnRH alone (same culture as previously reported Table 1—Fig. 3). Basal GtH levels (dose 0) are 27.21 ± 0.89 ng and 7.72 ± 0.64 ng/dish, respectively. Figure 4 shows sGnRH induced GtH release expressed as increase over basal levels. An overload of GtH did not modify sGnRH induced GtH release. GtH patterns are similar in control and experimental dishes: a significant increase in GtH release was obtained for sGnRH doses ranging from 10^{-8} to 10^{-6} M and the values obtained are not significantly different.

DISCUSSION

The use of different *in vitro* systems for studying the regulation of GtH secretion is now commonplace in mammals. In teleost fish some attempts have been made. Authors used either hemipituitaries in short-term incubation (carp: Breton *et al.*,

1971a, 1972) or whole organs in short term incubation (rainbow trout: Crim and Evans, 1980) and in organ culture (rainbow trout: Fåhræus-van Ree *et al.*, 1983). Recently pituitary fragments in a perfusion system have been described in goldfish (Mackenzie *et al.*, 1984). The use of dispersed pituitary cells of the whole pituitary has been also reported either in short-term incubation (rainbow trout: Fåhræus-van Ree *et al.*, 1982a,b) or in primary culture in a static model (rainbow trout: Fåhræus-van Ree *et al.*, 1982b; carp: Ribeiro and Ahne, 1982; Ribeiro *et al.*, 1983) and in a perfusion system (goldfish: Chang *et al.*, 1984). Recently a primary culture of purified pituitary gonadotrophs have been set up for African catfish (Leeuw *et al.*, 1984). The present paper describes a technique of dispersion and culture of pituitary cells in rainbow trout. The enzymatic collagenase procedure that we used produces a high yield of living cells which are firmly attached on Day 3 of culture and are able to be responsive to GnRH. Using another enzymatic dispersion technique, Fåhræus-van Ree *et al.* (1982b) have also obtained dispersed pituitary cells of rainbow trout able to be kept in primary culture and to be responsive to GnRH. In our study the response to GnRH is a function of incubation time, as already described for short-term

FIG. 3. Influence of the preincubation duration and of the cell density initially plated on the responsiveness of the gonadotrophs to various doses of sGnRH. Wells were initially plated with 6.2×10^4 cells or 3.1×10^4 cells and a 24-hr incubation with sGnRH was performed on Days 2, 3, and 5 after plating. Values are $\bar{x} \pm \text{SEM}$ ($n = 4$).

FIG. 4. Influence of an overload of 20 ng of GtH on cells responsiveness to various doses of sGnRH during the 24-hr period of an incubation performed 3 days after plating. Wells were initially plated with 6.2×10^4 cells. The results for sGnRH stimulated GtH release without a GtH overload come from the same cultures as shown in Fig. 3. Values are $\bar{x} \pm \text{SEM}$ ($n = 4$) and are expressed as GtH increase compared to basal level (GtH measured in doses 0 wells).

incubation of whole pituitary in trout (Crim and Evans, 1980) or for carp pituitaries in monolayer culture (Ribeiro *et al.*, 1983). We found that a 24-hr incubation period is necessary to observe a GtH release highly different from control wells. Furthermore, we demonstrated for the first time that GnRH stimulates GtH release in a dose-related fashion from isolated pituitary cells of rainbow trout. We observed a response to GnRH for doses ranging from 10^{-9} to 10^{-6} M of both tested peptides, the salmon GnRH and the mammalian LHRH analog (D-Ala⁶ LHRH). The average increase in comparison with basal release (dose 0) was approximately two and a half times higher. As the two tested peptides are active in the same dose range we think that they are equipotent in our *in vitro* system. To our knowledge our work is the second one comparing *in vitro* the action of mammalian and salmon GnRH on GtH secretion. The

first trials were made in goldfish with superfused pituitary fragments collected from postspawning males by Mackenzie *et al.* (1984). Because both peptides were active in the same dose range, elicited response profiles which were similar in time course and stimulated maximal responses of equal magnitude, these authors concluded also that the peptides are approximately equipotent in their system. However, *in vivo* in goldfish, Peter *et al.* (1985) have recently demonstrated that D-Ala⁶ LHRH is more active than sGnRH in terms of magnitude and duration of the GtH response. These authors suggest that *in vivo* the decreased degradation of LHRHa may be the primary factor determining its potency. As, in our static *in vitro* system, we did not study the degradation of both peptides as well as their pituitary receptor binding affinity during the 24-hr incubation period, we cannot determine the factors contributing to their equal efficiency.

As the gonadotrophs present in our primary cultures were sensitive to GnRH, we attempted to optimize the conditions of culture to increase the sensitivity of our system and to decrease the numbers of fish required. First the time was varied between plating and incubation with GnRH. Second, wells were initially plated with half the quantity of cells. It appears that the responsiveness of our system is dependent on the number of cells present in the dish at the time of GnRH incubation. In fact, when the density of cells was weak after either a low initial plating (3.1×10^4 cells) or when cells are not fully grown and firmly attached on Day 2 after an initial plating with 6.2×10^4 cells, only the highest dose of sGnRH (10^{-6} M) induced, at the term of the 24-hr period of incubation, an increase in GtH release. In both cases, the small number of cells was assessed by microscopic observation and corroborated by the low cells GtH content and basal GtH release in comparison with those observed in wells initially plated with 6.2×10^4 cells

and stimulated on Day 3. A longer duration of incubation could have been sufficient to increase the sensitivity of this *in vitro* system and to allow us to use a smaller amount of cells. However, by increasing the duration of incubation, we may have encountered some problems related to the age of the culture. Indeed, we noticed heterogeneous GtH release in control wells (dose 0) as well as in sGnRH treated wells when incubation with GnRH is performed 5 days after plating. As a consequence, this heterogeneity caused a decrease in the sensitivity of our system since only the highest dose of GnRH, i.e., 10^{-6} M, induced a significant increase in GtH release. However, by microscopic observation no changes in cell morphology were observed and the plates were not invaded by fibroblast proliferation as already described in mammals (O'Connor *et al.*, 1980). In mammals, it is well known that in monolayer culture, basal as well as LHRH stimulated LH and FSH release decrease with culture time (Vale *et al.*, 1972; Tixier-Vidal *et al.*, 1973). However, this decrease appears more than 1 week after plating. In our work, on Day 5 basal GtH release during the incubation period, although lower, is not significantly different from the one seen on Day 3. The fact that spontaneous GtH release as well as sGnRH induced GtH release are heterogeneous between replicates, leads us to think that this heterogeneity is not due to the damage of GnRH receptor. This heterogeneity could be due to a decrease in the synthesis capacity of the cells with time. Additional studies on the kinetics of the synthesis capacity of gonadotrophs present in the primary culture are necessary to confirm or refute this hypothesis.

Therefore, optimal conditions for studying the regulation of GtH secretion by GnRH in mature male rainbow trout in 96-well plates are initial plating with 6.2×10^4 cells, incubation with GnRH 3 days after plating and a duration of incubation of 24 hr. However, the presence of released GtH

in the medium for 24 hr could have interfered with the sensitivity of the system. As the pattern of sGnRH induced GtH release as well as the sensitivity of the system were not modified when we added GtH in the wells, we conclude that this static *in vitro* system is convenient for a certain type of studies on regulation of GtH synthesis. Preliminary work in progress, unreported here and conducted on males and females at different stages of the sexual cycle corroborate this fact since the gonadotrophs respond to GnRH in a characteristic pattern as predicted from *in vivo* experiments (Weil *et al.*, 1978). Further investigations are in progress to clarify this variable pituitary responsiveness to GnRH throughout the sexual cycle and to study the possible role of different steroids on its variation.

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