Studies on the release of gonadotrophins during the superfusion of isolated rat pituitaries in a continuous flow system

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Summary. A continuous flow system has been developed to study the endocrine functions of isolated organs. The procedure has been used to investigate the effect of a synthetic gonadotrophin-releasing hormone (Gn-RH) upon the anterior pituitary gland of the adult male rat. A Radiometer blood gas/pH analyser was used to monitor the pH and partial pressures of oxygen and carbon dioxide in the perfusion medium. A series of six experiments with four pituitaries per flask established that the rate of gonadotrophin release under basal conditions was 646 ± 301 (S.D.) ng LH/ml and 404 ± 124 (S.D.) ng FSH/ml. The duration and intensity of the response to Gn-RH was assessed by measuring the areas under the curves according to the trapezoidal rule. A significant increase in the release of FSH and LH was obtained by the administration of 250 ng Gn-RH/ml medium, and dose-response curves were produced for up to 10 μ g/ml.

Introduction

The ability of synthetic gonadotrophin-releasing hormone (Gn-RH) to release LH and FSH from the rat anterior pituitary has been demonstrated under a variety of experimental conditions (Schally *et al.*, 1972; Makino, 1973; Ondo *et al.*, 1973). There is still, however, a lack of detail regarding the biochemical mechanisms by which this release may be affected. Studies that have involved the administration of synthetic Gn-RH by infusion into the systemic or hypophyseal portal blood system have provided useful information, but suffer from the limitation that the results are influenced by many factors, including the feedback of gonadotrophins and steroids upon the hypothalamic-pituitary complex.

An alternative approach has been to study the effect of Gn-RH on the release of gonadotrophins from the isolated anterior pituitary maintained in a static medium. This procedural format, however, has the inherent problem that it allows the accumulation of metabolic products and their possible feedback effects. Accordingly, more recent developments have involved the adaptation of a superfusion system that was first devised for studies on the adrenal cortex by Tait *et al.* (1967), and subsequently applied to limited studies on anterior pituitary tissue from the rat (Serra & Midgley, 1970), quail (Smith & Follett, 1971) and cow (Zolman & Convey, 1972).

The present communication is concerned with a detailed description and evaluation of specialized equipment for the superfusion of rat pituitaries in a continuous flow system. In addition, results are presented which show that the administration of graded doses of Gn-RH produce well-defined, reproducible dose-response curves in terms of the release of LH and FSH, which should serve as a basis for subsequent studies on the mechanism of action of the releasing hormone.

Materials and Methods

Preparation of pituitaries

The animals used were male Wistar Rats weighing over 200 g. The rats were killed by decapitation and the pituitaries removed. On every occasion the removal of the pituitaries was completed between

09.30 and 10.00 hours. The pars posterior and median eminence were dissected away and discarded, and the remaining portion (pars anterior) was placed in the flow chamber. Four pituitaries per flask were used routinely in this study.

Continuous flow apparatus

The system was essentially the same as that described by Serra & Midgley (1970), but differed in the following aspects. The incubation flasks (Text-fig. 1) were made of perspex, and consisted of two identical halves that when bolted together formed a water-tight inner recess which contained a small perspex disc. This disc had four holes (4 mm in diameter and 4 mm deep) which retained the pituitary tissue. The number of tissues used in each flask can be easily modified by using a disc with a corresponding number of holes. A stainless-steel gauze support was placed on each side of the disc (mesh count 325, plain weave: R. Cadish & Sons, London). The inlet and outlet port of each flask was a female Luer fitting to which the infusion lines (Disposable Manometer Lines, Portex Ltd, Hythe, Kent) were connected. A three-way tap was incorporated into the infusion line in order to facilitate changes of medium or the administration of potentially active compounds. After assembly and connection, the incubation flasks were immersed in a water bath at 37°C.

Text-fig. 1. Schematic drawing with dimensions (mm) of the perspex incubation flasks used to retain the anterior pituitaries during the course of the infusion. (1) Luer fitting for connection to the infusion lines; (2) holes to enable the halves to be bolted together; (3) stainless-steel gauze support disc; (4) tissue-retaining inner disc with holes for four pituitaries.

A multichannel peristaltic pump (Gilson Minipuls II, Anachem Ltd, Luton) was used to maintain the flow of medium through the system at a rate of 250 $\mu\text{l}/\text{min}/\text{flask}$ and an automatic fraction collector (Ultrac 7000, LKB Instruments, Croydon, Surrey) was set to collect samples every 8 min (≈ 2 ml infusate). The infusion medium was Krebs-Ringer bicarbonate buffer with 0.2% (w/v) glucose (KRBG).

A preliminary series of experiments was performed using a Radiometer blood gas/pH analyser (V. A. Howe & Co. Ltd, London, S.W.6), to establish the partial pressures of oxygen and carbon dioxide and the pH of the medium before and after passage through the system. It was found that vigorous gassing with $\text{O}_2:\text{CO}_2$ (95:5 v/v) supersaturated the medium with O_2 (600 mmHg) and CO_2 (35 mmHg) and maintained the pH at 7.4, with the medium equilibrated at 37°C. Measurement of the same parameters after the medium had passed through the system gave the levels of P_{O_2} as 200 mmHg and the P_{CO_2} as 35 mmHg, the pH remained stable at 7.4. These figures show the gas tensions in the medium to be suitable, as the P_{O_2} in fresh rat blood is approximately 60 mmHg and the P_{CO_2} is about 30 mmHg, with a pH of 7.4.

The administration of a trace dose of ^{125}I at the three-way tap, and the subsequent collection and counting of the samples, established a clearance time of 5 min for the system. Consequently, after starting infusion of Gn-RH, the next collection tube to be completed was designated as time 0 min. The pituitaries were infused with KRBG for about 1.5 hr before the administration of releasing hormone. This preincubation period has been previously demonstrated as sufficient to allow the

pituitary tissue to equilibrate in the system and maintain a relatively constant basal release of gonadotrophins.

Gonadotrophin hormone-releasing hormone

Solutions of Gn-RH (Hoechst Pharmaceuticals 471, or I.C.I. 88,262) were prepared in KRBG buffer, and equilibrated with O₂:CO₂ (95:5 v/v) at 37°C. Selected doses (250 ng, 500 ng, 1000 ng and 10 µg/ml) were infused in a variable order, for a minimum period of 40 min. An interval of 40 or 48 min was allowed between each administration.

Sample collection and assay

All samples were frozen at -20°C after collection. LH and FSH were measured by radioimmunoassay. A 200 µl aliquot of the sample was taken for the determination of LH and a 100 µl aliquot for FSH; all samples were assayed in duplicate. The sample volume in the tube was adjusted to 400 µl in each case using phosphate-buffered saline (PBS), pH 7.4, containing 1% (w/v) bovine serum albumin (Fraction V, Sigma London Chemical Co. Ltd). The appropriate antigens (NIAMDD-Rat-LH-1-3 and -Rat-FSH-1-3) were labelled with ¹²⁵I using a modification of the Chloramine T method (Hunter & Greenwood, 1962). A 100 µl (≈ 20,000 ct/min) aliquot of labelled antigen was added to all tubes. The LH and FSH content were assayed using the NIAMDD-Anti-Rat-LH-S1 and -Anti-Rat-FSH-S6 antisera respectively. Separation of antibody bound from free radioactivity was performed using a double-antibody method. A 1:120 (v/v) solution of donkey anti-rabbit precipitating serum (RD 17, Wellcome Reagents Ltd, Beckenham) and 0.05 M-EDTA/PBS, pH 7.4, was prepared, containing 1:1200 (v/v) of non-immune normal rabbit serum (TC 69, Wellcome Reagents Ltd). A 200 µl aliquot of this solution was added to all tubes, except those used to determine the total radioactivity. After incubation and centrifugation (2000 g, 20 min, 4°C), the supernatant was decanted, and the tubes allowed to drain for 2 hr at room temperature. The radioactivity was then determined in an automatic γ-counter. The reference preparations were NIAMDD LH-RP-1 for LH, and FSH-RP-1 for FSH. The results were expressed as ng of the respective standards/ml of infusion medium.

Results

Evaluation of the radioimmunoassays

There was no significant cross-reaction of the antisera with either Gn-RH or the medium before infusion. Further evidence for specificity was obtained from the degree of parallelism between the standard curves of the respective hormones and serial dilutions of the medium after the tissue had been perfused.

The limits of sensitivity for the methods were defined as twice the standard deviation obtained from the measurement in triplicate of the total amount of radioactivity bound to antibodies in the absence of unlabelled material. The intra-assay variation was assessed by the analysis in duplicate of 50 samples of medium after the tissue had been perfused, and the inter-assay variation by replicate analysis of one sample over the course of 12 assays. These results are shown in Table 1. The range of values obtained for the determination of known amounts of hormone (100, 200 and 400 ng/ml) added to previously assayed fractions of the perfusate were within the intra-assay variation.

Basal release of LH and FSH

The concentrations of gonadotrophin released into the medium during the period of the incubation were determined. The range of values was 646±301 (S.D.) ng LH/ml, and 404±124 (S.D.) ng FSH/ml. The overall mean, and the mean deviation from the mean, for both gonadotrophins during six infusions of KRBG over a 4-5 hr period can be seen in Text-fig. 2.

Table 1. Evaluation of the radioimmunoassay methods for (a) LH and (b) FSH

	No. of determinations	Mean (range) (ng/ml)	Coefficient of variation (%)
(a) Precision			
Intra-assay variation	50	—	4.3
Inter-assay variation	12	121 (105–150)	9.5
Sensitivity	10	9.7 (8.9–12.2)	9.4
(b) Precision			
Intra-assay variation	50	—	3.7
Inter-assay variation	12	219 (190–240)	8.3
Sensitivity	10	12.5 (12.3–13.1)	3.6

Text-fig. 2. The basal gonadotrophin levels (mean plus mean deviation from the mean) of LH and FSH during the course of six 4–5 hr infusions with Krebs–Ringer bicarbonate buffer.

Effect of Gn-RH

A typical pattern of release for LH and FSH elicited by the repeated administration of 1 μ g Gn-RH/ml to the infusion system can be seen in Text-fig. 3. The areas below the curves for the release of LH and FSH were calculated by the method of the trapezoidal rule. Infusions of Gn-RH in the mass range of 250 ng to 10 μ g/ml produced a dose–response relationship, and the significance of the difference between these responses was determined by Student's *t* test. The results are shown in Table 2.

Table 2. Results from the statistical analysis (Student's *t* test) of the response areas of FSH and LH after administration of various quantities of Gn-RH

Conc. Gn-RH/ml medium	FSH area response (mean $\pm$ SE)	Significance	LH area response (mean $\pm$ SE)	Significance
1 250 ng	23 $\pm$ 3.5 (6)		28 $\pm$ 3.5 (16)	
2 500 ng	49 $\pm$ 7.0 (8)	$P < 0.01$ versus 1	46 $\pm$ 6.5 (12)	$P < 0.01$ versus 1
3 1000 ng	44 $\pm$ 8.2 (9)	$P < 0.05$ versus 1 N.S. versus 2	49 $\pm$ 5.0 (15)	$P < 0.0025$ versus 1 N.S. versus 2
4 10 μ g	63 $\pm$ 6.0 (2)	$P < 0.0025$ versus 1 N.S. versus 2 N.S. versus 3	77 $\pm$ 12.0 (2)	$P < 0.0005$ versus 1 $P < 0.05$ versus 2 $P < 0.05$ versus 3

Figures in parentheses indicate the number of incubations.

Text-fig. 3. The results obtained after assay of LH and FSH concentrations in the fractions collected after infusion of Gn-RH. The stippled bars represent the periods of Gn-RH infusion at 1 µg/ml medium.

Discussion

The pituitary infusion system reported by Serra & Midgley (1970), has been developed to enable single or multigland incubations. The ability to keep up to four pituitaries in the same flask ensured that, even if one or two suffered damage or thermal shock during removal, there was sufficient active material remaining to produce a response to Gn-RH.

The volume of each aperture of the tissue retaining disc was chosen to approximate the volume of the rat anterior pituitary. This design ensured that the infusing medium passing through the incubation flask must pass over and around the enclosed tissue, thus removing the necessity for agitating the flasks.

It has been reported (Porter & Smith, 1967) that the blood flow through the hypophysial portal vessels of an anaesthetized adult male rat ranges from 0.6–1.0 µl/mg tissue. Since the weight of the anterior pituitary varies from 7–12 mg, the total portal blood flow by computation is between 4.2–12 µl/min. Although only the outside of the gland was being infused with medium, it was found that too slow a flow rate caused very lengthy clearance times and prolonged incubation periods. The infusion rate was therefore increased to 250 µl/min/flask of four glands (62.5 µl/min/gland).

The results obtained from the measurement of the basal release of LH and FSH appears to indicate that the pituitary tissue requires 1.0–1.6 hr to equilibrate in the infusion medium. After an initial surge of FSH, possibly released from damaged pituitary cells, the tissue appears to stabilize at around 1 hr, and then recover slightly to maintain a relatively constant level of release for 5–6 hr. The pattern for the basal release of LH differs slightly. After a very large release of LH over the first hour, the mean level then falls gradually during the course of the infusion and at the end of about

5 hr has dropped to 30% below the overall mean. It could be that the release of LH is a more sensitive parameter of the physiological state of the pituitary than is the release of FSH. Notwithstanding this overall fall in LH release, the response to Gn-RH was consistently larger than that for FSH.

Within the mass range of Gn-RH administered (250 ng–10 µg/ml), a similar pattern of response for both LH and FSH was produced. The response to 250 ng/ml was the first significant release that could be discerned from the inherent background fluctuation in the concentration of LH and FSH. At the intermediary doses of 500 and 1000 ng/ml the response reached a plateau that was only exceeded by increasing the dose by a factor of 10 to 10 µg/ml.

The dose levels of Gn-RH required appear to be higher than those used for administration *in vivo* (Rippel *et al.*, 1973) and this finding merits further investigation. It could be explained by the relatively brief exposure time of the tissue to releasing hormone, or to the associated problems of gland, tissue and cellular permeability to Gn-RH and the gonadotrophins. In this connection the elevation of the basal levels of both LH and FSH in the medium after the initial infusion of Gn-RH could reflect either a delayed entry of Gn-RH into the inner tissue, or the subsequent release of gonadotrophins from this source.

Notwithstanding these limitations, the infusion system should prove useful for further studies on the mechanism of action of Gn-RH at the biochemical level. The results to date show that isolated pituitaries release LH and FSH in a controlled manner, and respond to graded doses of Gn-RH. Studies are in progress regarding the role of various gonadal steroids, cyclic nucleotides and prostaglandins, both in isolation and in conjunction with Gn-RH.

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