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A Model System for the Study of Luteinizing Hormone (LH) Secretion: Lack of Effect of Fetal Calf Serum on Gonadotropin-Releasing Hormone Stimulated LH Release[1] (41402)

JOAO L. C. BORGES,[2] DONALD L. KAISER, WILLIAM S. EVANS, AND MICHAEL O. THORNER

Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Abstract. Continuous perifusion of dispersed anterior pituitary cells recently has been applied to the study of LH secretion. We undertook a series of experiments to determine whether or not fetal calf serum (FCS) is a necessary or beneficial addition to medium perifusing cells in culture up to 43.5 hr. Male Sprague–Dawley rats provided pituitaries for two perifusion systems (columns) set up in parallel. The cells in one column were perifused on both Day 1 (first 19.5 hr) and Day 2 (subsequent 24 hr) with Medium 199, while the cells in the other column were perifused with Medium 199 on Day 1 and with Medium 199 with 2% FCS for the initial 18 hr on Day 2. On Days 1 and 2 cells in both columns were challenged with five doses of GnRH (1–100 n*M*) given as 1.5-min pulses in a random order. After 4-hr of perifusion, basal release of LH decreased gradually with time in a manner that fit a least-squares derived second-order polynomial function. Mean basal LH release (ng/ml/10^7 cells ± SEM) on Days 1 (9822 ± 2450) and 2 (7710 ± 2100) was no different ($P = 0.1$). Mean GnRH-stimulated LH release on Day 1 (1063 ± 144.3) was higher than on Day 2 (746 ± 143.7) ($P = 0.036$). GnRH-stimulated LH release on Day 2 was the same for the columns with (805.9 ± 41.9) and without (653.2 ± 190.8) FCS ($P = 0.9$); furthermore, the slopes of the dose–response curves were identical ($P = 0.9$). Thus, our results showed that, using continuously perifused dispersed rat anterior pituitary cells for up to 43.5 hr: (1) basal LH release does not change significantly; (2) GnRH-stimulated LH release becomes less with time but dose–response characteristics remain constant; and (3) preincubation with FCS appears unnecessary to maintain responsivity of cells. Furthermore, we propose that the quantitative methods used to analyze our data may prove useful to other investigators employing similar perifusion techniques, thus allowing comparison of results from various laboratories.

In vitro studies of hormone secretion by the anterior pituitary have classically utilized two techniques: static incubation of intact or hemisectioned glands and primary cultures of enzymatically dispersed cells. Within the past several years, another approach in which pituitary fragments (1–6) or dispersed cells (7, 8) are continuously perifused has been applied by some laboratories interested in pituitary physiology. Using dispersed cells either attached to cytodex beads (7) or suspended in an inert matrix (8), both basal and dynamic secretion of several pituitary hormones including adrenocorticotropic hormone (ACTH) (9), growth hormone (GH) (10), thyroid stimulating hormone (TSH) (11), and prolactin (12, 13) have been investigated. Recently, we and others have described preliminary results using continuous perifusion for the study of luteinizing hormone (LH) release (1–8, 14). In order both to extend our initial observations and critically evaluate the potential of this system for studies of gonadotrope function, we designed a series of experiments to: (1) verify that a constant basal release of LH can be achieved; (2) document both the reproducibility of LH release and response to repeated pulsatile administration of a given dose of gonadotropin releasing hormone

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(GnRH) and that a dose–response relationship exists; and (3) determine if fetal calf serum (FCS) is an essential or beneficial addition to the medium when cells are continuously perifused for up to approximately 2 days. Finally, we sought to develop a quantitative method with which to analyze data resulting from studies using such a cell perifusion system.

Materials and Methods. *Preparation of the cells and the perifusion system.* Male Sprague–Dawley rats (200–400 g) were housed in a controlled environment at 21° with 12 hr of light (0500 to 1700 hr) and had free access to food and water. Six rats provided anterior pituitaries for each perifusion chamber. After decapitation, the anterior pituitaries were quickly removed from the cranium, separated from the neurointermediate lobes, and placed in Earle's basic salt solution (EBSS; Gibco). Each gland having been cut into approximately 30 pieces, the EBSS was removed and replaced with 10 ml of a trypsin-containing EBSS solution (0.2%, w/v; trypsin: 206 U/mg, Worthington Biochemical). The tissue-containing trypsin solution was transferred to a dispersion apparatus consisting of a Teflon cup into which a motor-driven Teflon paddle was inserted. The cup was immersed in a 37° water bath and the solution was exposed to 95% O_2 and 5% CO_2. The cells were mechanically agitated by the Teflon paddle turning at 130 rpm. After 15 min the supernatant was poured into a 12-ml conical tube and the tissue remaining was resuspended in 10 ml of the trypsin-containing EBSS. The procedure was repeated twice more. The cell suspension was centrifuged for 20 min at 420 g, the supernatant was discarded, and the cell pellet was resuspended in 1–2 ml of EBSS. After an aliquot was taken for cell count and assessment of viability (normal: $4-11 \times 10^6$ cells/six rats; 95% viability based on trypan blue exclusion), the cells were mixed with 0.5 g Bio-Gel P2 (200–400 mesh, Bio-Rad) which had been preswollen overnight in 0.9% (w/v) saline and then equilibrated with EBSS containing 0.04% (w/v) Lima bean trypsin inhibitor (Worthington Biochemical). The mixture of cells and Bio-Gel was drawn up into a 2-ml syringe which served as the perifusion chamber. The perifusate entering the chamber consisted nine parts of Medium 199 (Microbiological Associates; containing penicillin 200 U/ml, streptomycin 50 μg/ml, amphotericin B 3.75 μg/ml, and gentamicin 100 μg/ml and gassed continuously with 95% O_2 and 5% CO_2) and one part of saline, mixed distal to a peristaltic pump (Ismatec SA) which was used to propel the perifusate (flow rate 0.43 ml/min) through the cell chamber. After allowing the cell–Bio-Gel mixture to settle (3 to 4 min) the syringe was disconnected from the inflow line and the excess liquid discarded, leaving a volume of 0.1 ml between the cell–Bio-Gel mixture and the syringe nozzle. The inflow line was then reconnected to the syringe which was placed in a 37° waterbath. Any gas which had come out of the solution was removed from the perifusate prior to reaching the chamber by a line which withdrew 10% of the volume. Saline or saline with GnRH was introduced into the system using an automatic sampler (Newton Instruments, Inc.). The outflow line was connected to a fraction collector (Fractionette Alpha 400, Buchler Instruments, Inc.), with which timed samples of eluate were obtained for hormone determinations.

Assays. All eluate samples were stored at 4° and assayed for LH within 24 hr. LH was measured by a double antibody radioimmunoassay using reagents provided by the National Pituitary Agency. The standard used was rat LH RP-I. All samples from each experiment were run in a single assay. The intra- and interassay variations were 6.5 and 9.4%, respectively, at a concentration of 50.8 ng/ml.

Experimental design. Two perifusion systems (referred to below as pituitary cell *columns*) were set up in parallel between 1800 and 1900 hr on three separate occasions (Fig. 1). For the initial 15 hr following dispersion, the cells were perifused only with saline and Medium 199. Ten-minute samples of eluate were collected. After this 15-hr period, the cells in both columns were exposed to five different doses of GnRH (1, 3, 10, 30, and 100 n*M*) given in a random

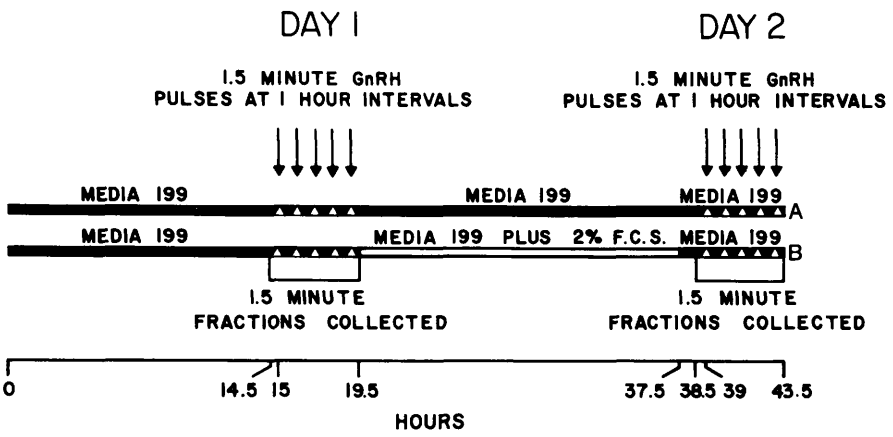

FIG. 1. Experimental design of the two perifusion systems. The cells in one system were perifused on both Day 1 and Day 2 (A) with Medium 199, while the cells in the other system were perifused with Medium 199 on Day 1 and with Medium 199 with 2% FCS for the initial 18 hr on Day 2 (B). On Days 1 and 2 the cells in both systems were challenged with five doses of GnRH (1–100 n*M*) given as 1.5-min pulses.

order as 1.5-min pulses at hourly intervals. During this time 1.5-min samples of eluate were collected. The initial 19.5 hr of the experiment (i.e., the 15 hr of saline and medium alone and the 4.5 hr of pulsatile GnRH exposure) were arbitrarily defined as Day 1. For the next 18-hr, the cells in one column (referred to below as column A) were exposed to saline and Medium 199 alone, while the cells in the other column (column B) were exposed to saline and to Medium 199 containing 2% FCS. At the end of this 18-hr period the FCS was withdrawn from the Medium 199 perifusing the cells in column B and 1 hr later the cells in both columns were exposed to pulses of GnRH in a manner identical to that on Day 1. The 19 hr of perifusion without or with FCS along with the 5 hr of intermittent GnRH administration were defined as Day 2.

Data analysis. Eluate LH (ng/ml/10^7 cells) measured during the 5-hr period of pulsatile GnRH challenge was plotted against time, thus generating four plots for each experiment (i.e., Days 1 and 2 of the two parallel columns). Visual inspection suggested no gross differences in LH release at a given dose of GnRH. Using a least-squares curve-fitting algorithm, a polynomial function was developed to define basal LH release for each column during the 5-hr periods of GnRH administration (15). Standard trapezoidal integration was employed to calculate the areas below (i.e., total basal LH release) and above (i.e., LH release in response to GnRH challenge) the baseline. Next, using general linear modeling (a technique similar to analysis of variance, but with more flexibility in defining the relationship among causal factors and the dependent effect) (16, 17) comparisons were made between mean basal LH release on Day 1 vs Day 2, mean GnRH-stimulated LH release on Day 1 vs Day 2, and GnRH-stimulated LH release on Day 2 (column A vs column B).

Results. During the initial 4 hr following dispersion of the cells, LH release declined rapidly. Thereafter, basal LH release, although decreasing with time, did so in a gradual and constant fashion defined at all points by the same polynomial expression (Fig. 2). The results of exposure of the cells to 1.5-min GnRH pulses on Days 1 and 2 are shown in Figs. 3 and 4. Mean basal LH release (ng/ml/10^7 cells ± SEM) on Day 1 (9822 ± 2450, $n = 6$) was no different from that on Day 2 (7710 ± 2100, $n = 5$) ($P = 0.1$). Mean GnRH-stimulated LH release on Day 1 (1063 ± 144.3, $n = 6$) was different from that on Day 2 (746 ± 143.7, $n = 5$) ($P = 0.036$). GnRH-stimulated LH release on

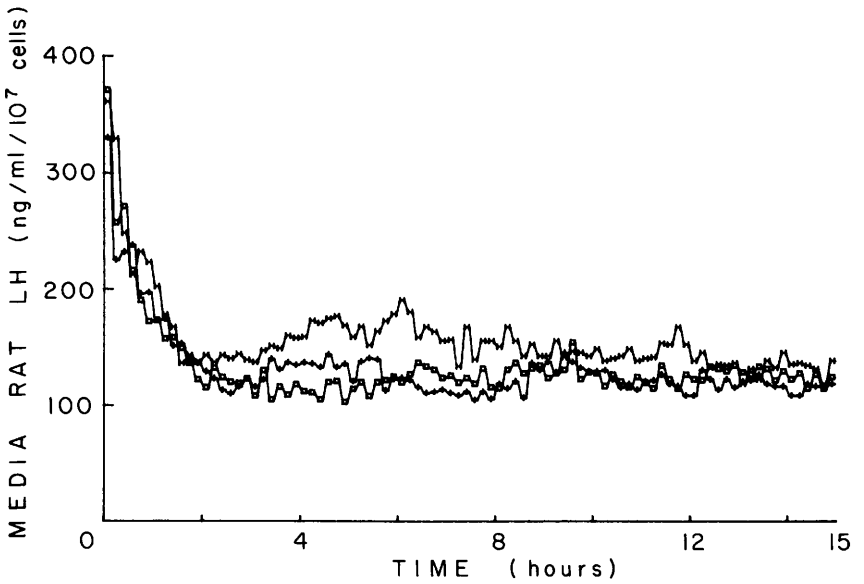

FIG. 2. The high initial release of LH (ng/ml/10^7 cells) with subsequent stabilization of the baseline by 3 to 4 hr is demonstrated.

Day 2, column A (653.2 ± 190.8, $n = 2$) was no different from that of Day 2, column B (805.9 ± 41.9, $n = 3$) ($P = 0.9$). The slopes of the dose–response curves on Days 1 and 2 were identical ($P = 0.9$). The Y intercepts on Days 1 and 2 were different ($P < 0.05$) reflecting lower GnRH-stimulated LH release on Day 2 (Fig. 5).

Discussion. Our objective was to further develop and validate an *in vitro* technique

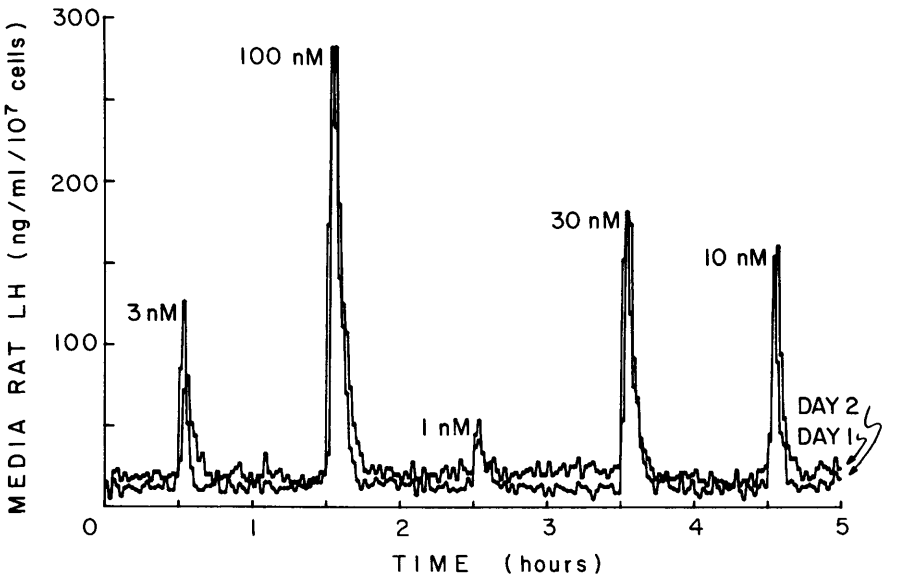

FIG. 3. Stimulation of LH release by varying doses of GnRH on Days 1 and 2 (B). In this experiment cells were exposed for the initial 18 hr of Day 2 to 2% FCS in Medium 199. The concentration of rat LH (ng/ml/10^7 cells) in perifusion eluate is shown.

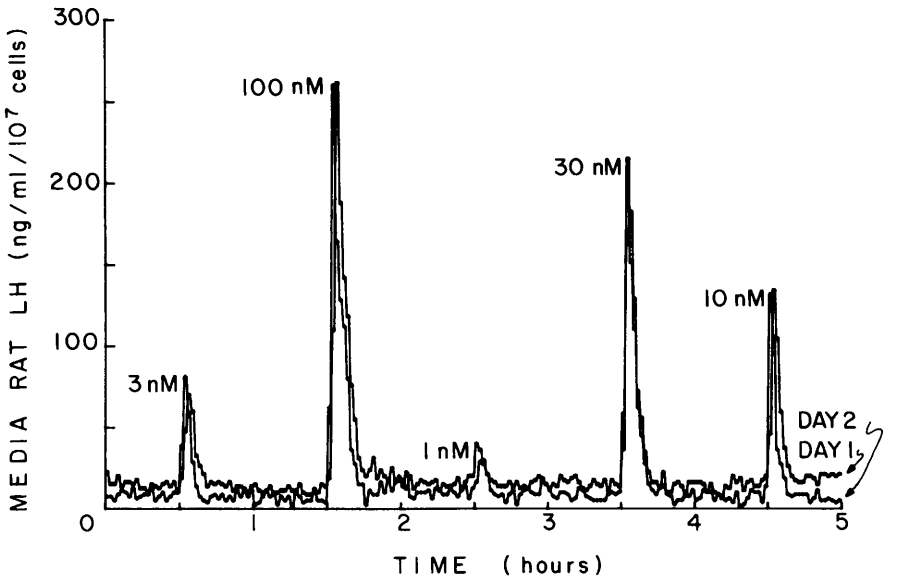

FIG. 4. Stimulation of LH release by varying doses of GnRH on Days 1 and 2 (A). In this experiment cells were exposed overnight to Medium 199 alone. The concentration of rat LH (ng/ml/10^7 cells) in perifusion eluate is shown.

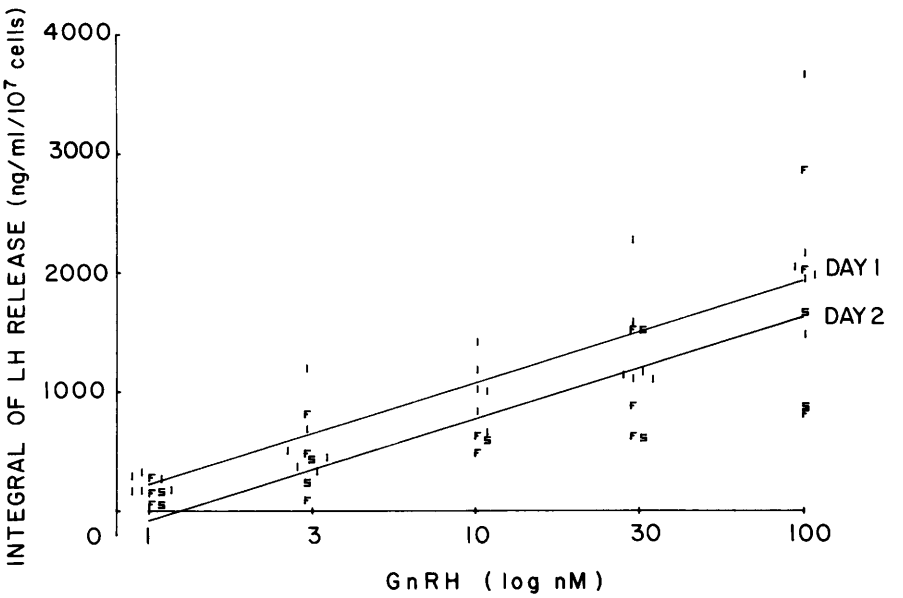

FIG. 5. Relationship between integral of LH released from pituitary cell columns and logarithm of GnRH dose is shown. Note that slopes of dose–response curves on Days 1 and 2 are the same. Eighteen hours of exposure to FCS did not alter dose response on Day 2. F, Responses on Day 2 of cells exposed to 2% FCS (B). S, Responses on Day 2 of cells exposed to Medium 199 alone (A). I, Responses of cells on Day 1.

for investigating basal and stimulated LH release using a dispersed anterior pituitary cell perifusion system. Such a system offers potential advantages when compared to other *in vitro* techniques. Static incubation of anterior pituitary glands may have the problems of poor diffusion of nutrients and stimulatory/inhibitory agents into all cells as well as necrosis of the central tissue with time and poor reproducibility of results. Primary cultures of dispersed cells may well overcome the difficulties of diffusion, necrosis, and reproducibility of results, but do not allow the intermittent, pulsatile (over seconds or minutes) administration of stimulatory/inhibitory agents (such as GnRH) which more accurately simulates the *in vivo* situation (18–22). Continuous perifusion of dispersed cells allows brief as well as prolonged exposure to stimulatory/inhibitory agents; furthermore, continuous perifusion diminishes concerns about accumulation of proteolytic enzymes and secretory products which theoretically may alter detectability of release of hormone. Another advantage of dispersed cells in the perifusion system compared to monolayer cultures concerns the time required between removal of the gland from the animal and initiation of the experimental protocol. Due to the need for the cells to adhere to the culture dish and possibly time for the receptors to recover from enzymatic damage occurring during dispersion, studies are most often begun 24 to 48 hr or more after plating in the primary culture system. A similar time lag is required in perifusion techniques in which cells are allowed to adhere to cytodex beads (7). In contrast, we have shown previously that cells in our system are GnRH responsive within 4 hr after dispersion, thus allowing experiments to commence within a few hours following removal of the gland from the donor animal.

Kao and colleagues (1) and Loughlin and colleagues (8), using continuous perifusion of dispersed cells, have observed a linear dose–response curve between 0.7 and 23.5 and 1 and 50 n*M* GnRH, respectively. We have confirmed these findings and have in addition demonstrated a similar relationship up to and including 100 n*M* GnRH (the maximum dose tested). Furthermore, we have shown clear stimulation of LH with 1.5-min pulses of GnRH. To our knowledge, no one has previously documented definite LH release in response to this brief a pulse of GnRH. We found it of interest that, although LH release in response to GnRH was less on Day 2, the characteristics of the dose–response curve, other than the expected change in the *Y* intercept, remained the same.

Preincubation of dispersed cells in FCS has been claimed to be an essential requirement by some investigators. In addition to being expensive and in short supply, FCS contains a variety of factors of which many are poorly defined (23). Moreover, FCS contains gonadal steroids that have been shown to vary in concentration among batches. In studies on gonadotropin secretion, the gonadal steroid content of the perifusate must be known, as such steroids may influence both basal and stimulated gonadotropin release. For these reasons, we felt it important to document whether or not basal or GnRH-stimulated LH secretion was enhanced in cells perifused in FCS containing medium compared to medium alone. No such enhancement was seen in our experiments. It could be argued that the omission of FCS during the initial 15 hr of perifusion of the cells could have been deleterious to the cells and that this "damage" was not reversible by exposure of the cells to FCS from 19.5 to 37.5 hr. This possibility, however, appears unlikely in view of a recently reported study utilizing a similar system, but in which the cells were exposed to FCS for the initial 24 to 72 hr (8); results from this study differed little from those reported here. We conclude that FCS is not an essential ingredient for cells perifused at least up to 43.5 hr.

Since various approaches have been employed to analyze data obtained from studies using perifusion systems, comparison of results from different laboratories has been difficult. We have developed an analytical approach which we hope may be of use to other groups utilizing similar perifusion methodology. Stimulation or in-

hibition of a hormone in the dispersed cell perifusion system is relative to basal release of the hormone. For LH secretion we have shown that, after approximately 4 hr, basal release declines very gradually and, at least for 43.5 hr, this baseline can be defined by a single least-squares-derived polynomial. Integration above the baseline provides a precise and unbiased method for measuring stimulated LH release. Finally, statistical analysis using general linear modeling is more powerful than standard analysis of variance, specifically allowing greater latitude in assessing relationships between causal factors and dependent effects.

In summary, we have demonstrated that continuous perifusion of dispersed cells lends itself well to the study of LH secretion by the anterior pituitary. The gonadotropes are responsive within a few hours after removal from the rat, release LH in a reproducible manner over a wide range of concentrations of GnRH given in a pulsatile fashion, and do not require FCS for at least 43.5 hr in culture. Data from this system can be analyzed in a simple but objective manner which will allow comparison of studies from various laboratories using similar methodology.

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