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Cycle-related LHRH responsiveness of superfused pituitary cells in a Phenol red free medium

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Anterior pituitary cell cultures are frequently used in studying the control of gonadotropin secretion. Historically, many (if not most) of these studies have been performed in the presence of Phenol red as a pH indicator. Phenol red preparations, because of their potential estrogenic activity, may have influenced the results of previous studies defining the relative luteinizing hormone releasing hormone responsiveness of rat anterior pituitary cells derived from various stages of the estrous cycle. We therefore felt it of interest to investigate this possibility by repeating our previous cycle-related superfusion studies [(1988) Life Sci. 42, 61–72] in the absence of these Phenol red preparations. Comparisons of data obtained in the presence or absence of Phenol red revealed cells derived from late proestrous (19.00) and cultured in the absence of Phenol red continued to evidence the highest LH responsiveness. However, diestrous 1 08.00 cells cultured in the absence of Phenol red were lower in responsiveness than previously observed in the presence of the substance and the responsiveness of proestrous 08.00 and 15.00 in the presence was lower in comparison to the same stages in the absence of Phenol red. The results suggest that Phenol red preparations are capable of modulating LHRH responsiveness in superfusion and that the effect is more pronounced at certain cycle stages than at others.

Pituitary; Gonadotropin; Estrogen

1. INTRODUCTION

Anterior pituitary culture systems have been widely used as tools in studying the modulation of gonadotropin secretion [1–7] and many of these studies have been conducted in medium containing Phenol red preparations (PR) as pH indicators; however, recent reports have suggested that these PR preparations are capable of exhibiting estrogenic activity [8–11]. Luteinizing hormone releasing hormone (LHRH) responsiveness in anterior pituitary tissue is modulable by estrogens both in vitro [12–19] and in vivo [20–25]. Indeed, estrogen emanating from the maturing follicle triggers the preovulatory gonadotropin surge [22,23,26–30]. Preliminary studies indicated that commercially prepared culture medium containing preparations of PR at concentrations of ~38 μM caused a significant elevation in LHRH stimulated LH release thereby confirming that our culture system was capable of exhibiting the PR effect described by others. Considering this observation, the goal of the current studies was to determine whether previously reported cycle-related studies performed by ourselves [1] and possibly those performed by others might have been influenced by the presence of PR preparations in culture media.

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2. MATERIALS AND METHODS

2.1. *Animals*

Female Sprague-Dawley rats were purchased from Holtzman Co. (Madison, WI) at 50 days of age and maintained under a 14:10 h (light:dark) cycle. For superfusion studies, animals evidencing 2 successive 4 day estrous cycles were utilized. Pituitaries were collected at 08.00 h on each day of the cycle as well as at 15.00 and 19.00 h proestrus (P0800 = proestrus 08.00; P1500 = proestrus 15.00; P1900 = proestrus 19.00; E0800 = estrus 08.00; D1 0800 = diestrus 1 08.00; D2 0800 = diestrus 2 08.00). Animal protocols were approved by the Institutional Committee on Animal Use.

2.2. *Preparation of growth medium*

The culture medium was composed of Dulbecco's Modified Eagle medium (DMEM) either with (Gibco 320-1965) or without (Gibco 430-3000EB) PR and supplemented with 5% fetal bovine (Difco no.5065-67) and 5% horse serum (Difco no.5357-67). This medium was referred to as complete growth medium (CGM); when PR was present, its final concentration in CGM was ~38 μM.

2.3. *Dispersal of pituitary tissue*

The dispersal procedure has been described previously [7,33].

2.4. *Cell culture system*

For superfusion, Cytodex beads from Pharmacia (no.17-0448-01) were utilized as described previously [33,36]. Columns were superfused for 5 h in DMEM, the first hour was allowed for basal release. At the start of the next four 1 h intervals, the cells were subjected to a single 100 μl PBS + gel (0.01 M phosphate + 0.1% gelatin) pulse carrying 4, 4, 4 and 100 ng LHRH, respectively; the 100 ng bolus was to test terminal responsiveness. We have described [1,33] the elution profiles of LHRH from this system. Control cultures received PBS + gel without LHRH. Fractions of 1 ml vol. were subjected to LH and FSH radioimmunoassay (RIA) utilizing reagents from the National Pituitary Hormone Distribution Program as described [7,34].

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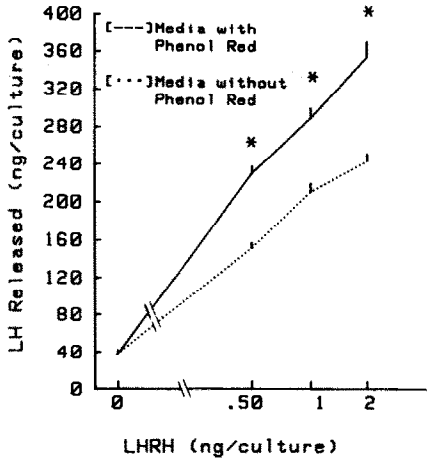

Fig.1. LHRH induced response of a common pool of dispersed anterior pituitary cells derived from randomly cycled 60–70-day-old intact female rats cultured in the presence and absence of Phenol red (~38 μM) for 3 days. The plotted data represent the mean ± SE of triplicate cultures.

2.5. *Evaluation of data*

Five anterior pituitaries were utilized per superfusion column and 6 columns were utilized per estrous cycle stage; each group of 5 rats was processed as an individual column. The mean effluent data for the 6 columns at each cycle stage were first plotted as ng LH or FSH per ng DNA [37]. Each of the 6 columns at each stage was individually plotted as ng LH or FSH per ng DNA; the PC Pulsar program [38,39] identified episodes of pulsatile LH and FSH response and a Jandel digitizer (Corte Madera, CA) was used to measure area (mm^2) under each identified pulse. The 6 columns were each exposed to three 4 ng LHRH pulses; since there was no statistically significant difference between the responses to these 3 pulses within a given cycle stage, the responses were averaged in order to make statistical comparisons between cycle stages. These data were evaluated by ANOVA and Duncan's Multirange test as we have described previously [1,33].

3. RESULTS

Examination of LHRH dose-response curves performed using monolayer cultures derived from a pool of commonly dispersed cells revealed significantly

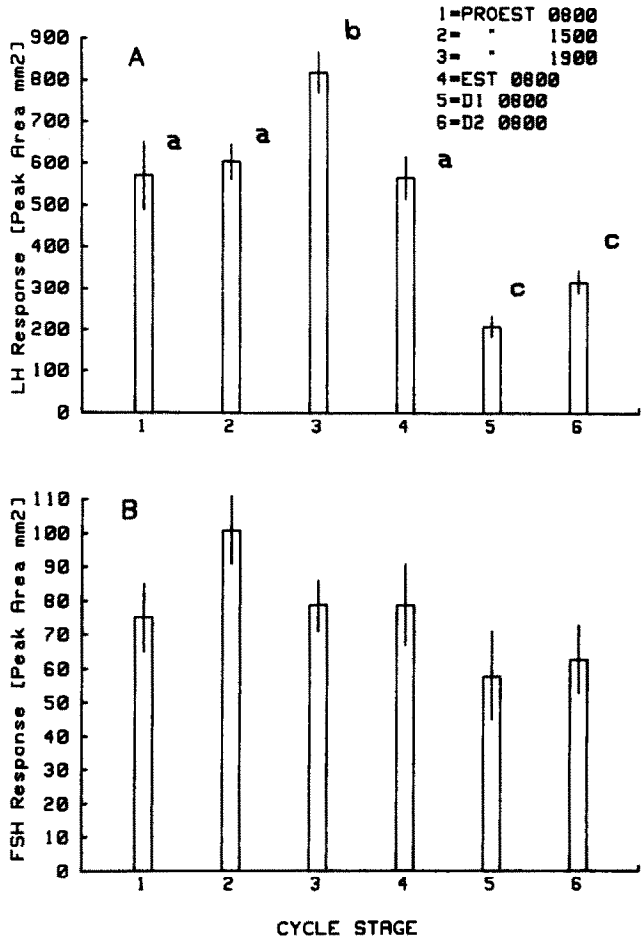

Fig.2. Statistical comparison of LH (A) and FSH (B) response to pulsatile LHRH observed in the absence of PR with superfused anterior pituitary cells derived from 60–70-day-old rats at proestrus 08.00, 15.00 and 19.00 as well as 08.00 estrus, diestrus 1 and diestrus 2 (bars 1–6, respectively). Each bar represents the mean of the responses to three 4 ng LHRH pulses delivered to 6 individual superfusion columns. Like letters (a,b,c) indicate stages which are not significantly different. Proestrus 19.00 was significantly larger than all other stages (bar 3 vs 1 ($P < 0.05$), vs 2 ($P < 0.01$), vs 4 ($P < 0.05$), vs 5 and 6 ($P < 0.01$)).

Table 1

LH and FSH response to pulsatile LHRH in superfused anterior pituitary cells derived from rats in specific stages of the estrous cycle

Cycle stage	LHRH pulse number							
	LH				FSH			
	1	2	3	4	1	2	3	4
DI 0800	208 ± 27	169 ± 38	157 ± 46	564 ± 148	70 ± 23	91 ± 20	43 ± 14	108 ± 26
DII 0800	260 ± 59	250 ± 56	255 ± 50	403 ± 57	57 ± 15	61 ± 14	68 ± 14	49 ± 10
EST 0800	572 ± 61	509 ± 94	487 ± 113	1023 ± 167	131 ± 40	67 ± 14	94 ± 25	127 ± 27
PRO 0800	706 ± 189	476 ± 46	451 ± 49	867 ± 146	87 ± 20	53 ± 9	106 ± 12	120 ± 22
PRO 1500	531 ± 73	616 ± 107	505 ± 57	762 ± 65	97 ± 17	106 ± 17	119 ± 21	84 ± 12
PRO 1900	908 ± 49	851 ± 115	705 ± 54	1021 ± 76	80 ± 13	89 ± 16	70 ± 12	99 ± 18

Pituitaries were derived from 60–70-day-old rats at the estrous cycle stages indicated. The data represent area (in mm^2) beneath pulsatile responses as calculated by a Jandel digitizer and software (Corte Madera, CA). The LHRH pulse regimen employed is described in section 2.4. The data are the mean ± SE of 6 individual superfusion columns utilizing 5 dispersed anterior pituitary glands/column.

greater responses in cultures incubated in media containing 38 μM PR; there was no significant difference in the DNA content of cultures grown in the presence or absence of this PR preparation (fig.1). All LHRH pulses resulted in pulsatile LH response regardless of cycle stage (table 1). Comparative LH responsiveness (fig.2A) was ranked as P1900 > P1500 = P0800 = E0800 > D2 0800 = D1 0800. There was no significant difference between P1500, P0800 and E0800; all were significantly lower than P1900 ($P < 0.01$, 0.05 and 0.05, respectively) and significantly larger than D1 ($P < 0.01$) and D2 0800 ($P < 0.01$). For FSH, all LHRH pulses induced pulsatile FSH release detectable by PC Pulsar (table 1). In terms of magnitude of response, the stages were ranked P1500 = E0800 = P1900 = P0800 = D2 0800 = D1 0800; ANOVA indicated there was no significant difference between any of these values (fig.2B).

4. DISCUSSION

Estradiol and progesterone are capable of independently and cooperatively modulating gonadotropin secretion through hypothalamic and pituitary effects [40]. Estradiol is of interest because its secretion from the developing follicle is the active trigger for the preovulatory gonadotropin surge [22,23,27,30]. Reports of potential estrogenic activity of PR preparations [8–11] in combination with the fact that estrogens are potent modulators of gonadotropin secretion led us to question whether PR preparations might have played a role in the relative ranking of responsiveness established in our previous studies (P1900 > E0800 > D1 0800 > P0800 = P1500 = D2 0800; no significant difference in P0800, 1500 and D2 0800; all were significantly smaller than P1900 ($P < 0.01$), E0800 ($P < 0.05$) and D1 0800 ($P < 0.05$)) and potentially in the studies of others. To determine this, we first confirmed that PR preparations modulated LHRH responsiveness in pituitary cells dispersed according to our protocol as indicated by significantly greater LHRH induced LH response in the presence of PR preparations. In view of these results, we proceeded to repeat in the absence of PR our previous superfusion studies which had been performed originally in the presence of PR preparations [1,33]. In these current studies, responsiveness was ranked P1900 > P1500 = P0800 = E0800 > D2 0800 = D1 0800; the data therefore indicated that cells cultured in media without PR preparations continued to show pulsatile LH release in response to pulsatile LHRH as well as the elevated responsiveness associated with proestrus. However, marked differences in relative LH response could be detected in certain non-proestrus stages. Cells collected at D1 0800 and cultured in media without PR were found to be lowest in LH responsiveness; in contrast, cells from this same cycle stage when cultured with PR preparations [1] were found to be highly responsive to LHRH. This time point corresponds in vivo to a period of low E2 and low LH secretion; it might be suggested then that incubation with PR preparations for the 48 h period utilized in previous studies partially substituted for in vivo E2 exposure thereby increasing LHRH responsiveness.

Another interesting comparative difference involved P0800 and P1500. In earlier studies in the presence of PR preparations [1], these stages were found to be non-significantly different from D2 0800 (which exhibited the lowest responsiveness); however, in the absence of PR, the P0800 and 1500 stages exhibited elevated responsiveness such that they were significantly larger than D1 and 2 0800. In vitro studies with anterior pituitaries derived from presurge proestrus rats have demonstrated that incubation of these cells with exogenously added estrogen results in a 50% reduction of LH secretion in response to pulsatile LHRH as compared to control cultures [15]. Although PR is a low affinity estrogen, it is present in DMEM at concentrations which would be high on a physiological basis. Therefore, the P0800 and P1500 stages cultured in the presence of PR may have exhibited diminished responsiveness due to estrogenic activity of the PR preparation present in the culture medium.

Our studies indicated that 48 h incubation of D1 0800 anterior pituitary cells in the presence of PR preparations resulted in a stimulation of LHRH induced LH release; this stimulatory effect of the PR preparation was not seen in cells recovered during D2 0800. This difference in these two cycle stages could be due to the prior existing in vivo estrogen and progesterone levels to which the pituitary cells had been exposed. Estrogens are known to alter the secretory dynamics of the pituitary. Rises in circulating levels are responsible for both the negative feedback suppression of LH and FSH secretion and for the abrupt preovulatory gonadotropin surge. Thus the variable effects of PR preparations on particular cycle stages in response to LHRH could be the result of in vivo exposure to gonadal steroids combined with exposure to PR preparations in culture.

Other investigators have reported that PR preparations at concentrations found in commercially prepared media were capable of eliciting changes in cellular response in a fashion similar to that of estrogen; these changes include increased cell proliferation and progesterone receptor synthesis in MCF-7 cells [8], stimulated Prl synthesis and secretion and LHRH induced LH release in rat anterior pituitary cells [11,14] in a time-frame which mimics that of estrogen [11,42]. Our data not only confirm that PR preparations are capable of exerting significant estrogenic action at the anterior pituitary cell but also indicate for the first time that the effects may be more strongly expressed at certain cycle stages than at others and that PR preparations should probably be avoided in pituitary cell

culture systems. To our knowledge, this is the first report: (i) to study potential PR effects on the LHRH responsiveness expressed in superfused anterior pituitary cell cultures; (ii) to carry out such studies at multiple stages of the estrous cycle; (iii) to report both LH and FSH data from the same samples; and (iv) to show that the PR effects, like those of estrogen, are more strongly expressed at specific cycle stages.

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