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Does Time of Exposure to Estradiol and LHRH Effect LH Release From Bovine Pituitary Cells?[1] (40304)

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Luteinizing hormone releasing hormone (LHRH) induced increase in serum LH is greatest coincident with periods of increased estrogen secretion in cows (1), ewes (2), women (3, 4) and female rats (5, 6). Exogenous estrogens also increase magnitude of LH release by LHRH in cows (7), ewes (8), women (9) and female rats (10). However, attempts to demonstrate direct effects of estradiol on LH secretion *in vitro* have yielded variable results, i.e. estradiol increased (11, 12), decreased (13–15) or did not change (16, 17) quantity of LHRH induced LH release *in vitro*.

In experiments reported, we investigated effects of estradiol and LHRH on LH secretion by bovine pituitary cells in primary culture. Variables were dose and time of exposure of cells to E_2 and LHRH alone or in combination.

Materials. Medium for culture was Dulbecco's minimal essential medium[3] supplemented with essential and non-essential amino acids and buffered as in reference 12. Stock solutions of synthetic LHRH[3], prepared in 0.1% knox gelatin:0.05 *M* phosphate buffered saline, were added to cultures in 10 μl vol. Estradiol-17β (E_2)[3] in 10% ethanol, was added in volumes such that final concentration of ethanol in medium was 0.1%.

Cell cultures. Bovine pituitary cell cultures were prepared (12). Briefly, bovine anterior pituitaries were sliced ($\cong$1 MM), diced ($\cong$1 MM^3) and resulting pieces washed thrice with medium. Pituitary cells were dispersed from these pieces by stirring in 0.3% collagenase[3] for 45 min then 0.25% Viokase[3] for 15 min. Washed cells were suspended ($\cong 5 \times 10^5$ cells/ml) in medium containing 10% bovine serum[3] and 1 ml of suspension transferred to each well of multiwell culture plates. Pituitary cells were in culture for 5 days with medium changed at 24-hr intervals beginning at 48 hr. On day 5 cells were washed 4 times with serum free medium and treatments begun. Medium did not contain serum during treatment.

Experimental design. Experiment 1. The objective was to determine effects of varying time of exposure and concentration of LHRH on quantity of LH released. Treatments were arranged as a five × six factorial experiment with concentration of LHRH (0, 0.1, 1.0, 10 and 100 ng/ml) and time (.75, 1.5, 3, 6, 12 and 24 hr) as main effects. There were six replicates per treatment combination (n = 180).

Experiment 2. The objective was to determine effects of varying time of exposure and concentration of estradiol on quantity of LH released. Treatments were arranged as a three × four factorial experiment with concentration of estradiol (0, 5 and 50 ng/ml) and time (3, 6, 12 and 24 hr) as main effects. There were 12 replicates per treatment combination (n = 144).

Experiment 3. The objective was to examine the interaction of estradiol and LHRH on LH release over time. Treatments were arranged as a four × two × five factorial experiment with concentrations of estradiol (0, 0.5, 5.0, and 50 ng/ml) and LHRH (0 and 100 ng/ml) and time of exposure to estradiol and LHRH (1.5, 3, 6, 12, and 24 hr) as main effects. There were four replicates per treatment combination (n = 160).

Within each experiment, treatments were begun concurrently and medium collected

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[3] Dulbecco's medium from Difco Labs, Detroit, MI; LHRH courtesy of Dr. R. Rippel, Abbott, N. Chicago, Ill.; Estradiol 17β and collagenase (type I-150 μ/mg) from Sigma, Chicago, Ill.; Viokase from GIBCO, Grand Island, New York.

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and frozen after the prescribed interval of treatment. Medium was assayed for LH by methods described in 18.

Statistical analysis of data. In instances where data, hormone concentrations or time were not normally distributed, statistical analysis were performed after logarithmic transformation of values. Data from each experiment were analyzed by analysis of variance appropriate to factorial experiments (19). Significant differences due to main effects were determined by Dunnett's t-test (20). Additionally, data were subjected to polynomial regression analysis (19) to evaluate change in LH release over time or concentration of hormones tested.

Results. Experiment 1. Effects of varying time of exposure and concentration of LHRH on LH concentration in medium are in Fig. 1. In the absence of LHRH, LH accumulated in medium during 24 hr and this increase was curvilinear ($P < 0.001$) i.e. rate of accumulation increased with time. Within time periods, increase in LH release by LHRH over the range 0.1 to 100 ng/ml, was linear ($P < 0.001$) when exposure was for .75, 1.5, 3, 6, or 24 hr but curvilinear ($P < 0.001$) when for 12 hr. Dose-response slopes generated from data normalized by logarithmic transformation were not different among times i.e. with the exception of 12 hr, LH release induced by 100 ng LHRH/ml was twice that of comparable control values. However, the actual increase in amount of LH release (ng/ml) over controls, induced by each concentration of LHRH, increased with increasing time of exposure.

Experiment 2. Effects of varying time of exposure and concentration of estradiol on quantity of LH in medium are in Table I. Estradiol did not affect concentration of LH in medium when present for 3 hr but increased ($P < 0.001$) LH relative to controls when present for 6, 12 or 24 hr. Both concentrations of estradiol tested increased LH accumulation in medium and magnitude of increase was dependent on the dose of E_2 i.e. 50 ng E_2 released more LH than 5 ng ($P < .01$).

Experiment 3. Effects of varying time of exposure and concentration of estradiol on LHRH induced LH release are in Fig. 2. Within each combination of LHRH and estradiol, accumulation of LH in medium was curvilinear ($P < 0.001$) and greater ($P < 0.001$) for cultures incubated with LHRH than for comparable controls. Estradiol, present for 1.5 or 3 hr, did not affect LH concentration in medium of cultures incubated with or without LHRH. However, when estradiol was present 6, 12 or 24 hr LH accumulation in medium was increased ($P < 0.001$) relative to controls. This was true for cultures incubated with or without LHRH. In addition, magnitude of LH release, within these time periods, was linearly ($P < 0.001$) related to concentration of estradiol used. A comparison of cultures incubated with and without LHRH, within time, revealed that slopes of estradiol dose-response were not different ($P > 0.10$).

Discussion. Results confirm our previous

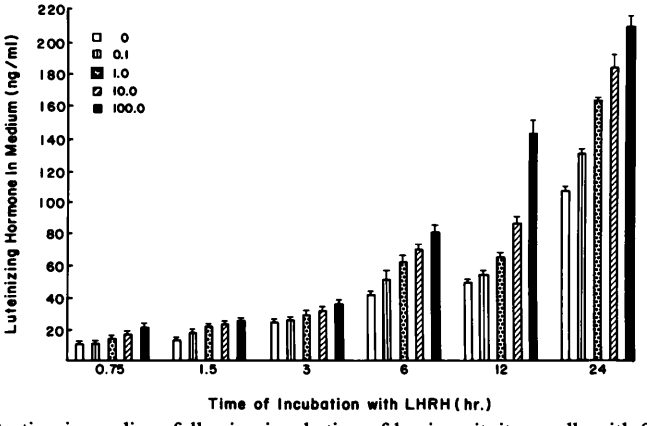

FIG. 1. LH concentration in medium following incubation of bovine pituitary cells with 0, 0.1, 1, 10 or 100 ng LHRH/ml media for .75, 1.5, 3, 6, 12 or 24 hr. Values are means ± SE.

observation that LHRH causes LH release from bovine pituitary cells in culture and that quantity of LH released is linearly related to concentration of LHRH over the range 0.1 to 100 ng/ml (12). This result was demonstrable when time of exposure to LHRH was as short as 45 min or as long as 24 hr. Additionally, percent increase in LH release relative to controls induced by each dose of LHRH was independent of time LHRH was present. This leads to the conclusion that ability of LHRH to induce LH release appears to be consistent for at least 24 hr. Resolution of effects of LHRH on increasing LH concentration, as determined by difference in LH concentration of control cultures and those given LHRH, increased markedly with time. For this reason, time of exposure to LHRH greater than 3 hr may be desirable.

Present results confirm our previous observation that estradiol when present for 24 hr increased basal and LHRH induced LH release from bovine pituitary cells (12). These experiments provide evidence that estradiol must be present for more than 3 hr before stimulatory effects on LH release are demonstrable. Our failure to demonstrate an effect of estradiol at .75 or 3 hr agrees with results of others using rat pituitary cells in culture (14). This lag period may represent time required for estradiol to exert biological changes in gonadotrophs that result in increased LH release. Inhibitors of protein synthesis block the stimulatory effect of low doses of estradiol on LH release (17). Failure to detect an effect of estradiol during the first 3 hr of treatment may reflect time required for protein synthesis. Alternatively, this lag may be an artifact of the culture system.

TABLE I. EFFECT OF ESTRADIOL-17β AND TIME OF EXPOSURE TO ESTRADIOL ON MEDIUM LUTEINIZING HORMONE LEVELS.

Time (b)	Estradiol-17β (ng/ml)* 0	5	50	Avg
3	8.6 ± .7^a	9.2 ± .6^a	9.2 ± .5^a	9.0
6	14.3 ± .9^a	21.3 ± .9^b	23.5 ± .9^b	16.4
12	22.5 ± 3.7^a	32.3 ± 3.6^b	41.7 ± 2.8^c	32.2
24	64.4 ± 2.4^a	80.7 ± 2.7^b	100.8 ± 4^c	82.0
Avg	27.5	35.9	43.8	

* Means within time periods with different superscripts are significantly different at $P < 0.05$. Values are means ± SE ($n = 12$).

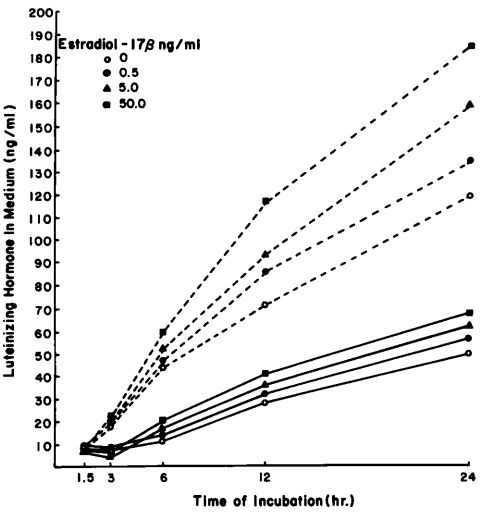

FIG. 2. LH concentration in medium of bovine pituitary cultures incubated with 0, .5, 5 or 50 ng/ml E_2 for 1.5, 3, 6, 12, or 24 hr with LHRH (0 or 100 ng/ml) present through out the incubation period. Dashed lines represent data obtained when LHRH was present.

Considering that rate of accumulation of LH in medium accelerated during the 24 hr experimental period, failure to detect an effect of LH release at 45 min and 3 hr may be because LH release at this time is very low and gonadotrophs not receptive to this stimulus. An argument against the latter view is that LHRH was equally efficacious in causing LH release at all times tested. Our results also demonstrate that once estradiol affects LH release, this effect remains quantitatively similar at least to 24 hr in cultures incubated with and without LHRH. LH release by rat pituitary cells was increased by 500 ng/ml estradiol for 6 or 24 hr (15) or 0.27 ng/ml for 40 hr (11).

Results of experiments designed to investigate *in vivo* effects of estradiol on LHRH induced LH release revealed a biphasic effect i.e. estradiol first decreased, then increased magnitude of LHRH induced increase in serum (21–23). In these *in vitro* experiments, estradiol did not inhibit basal or LHRH induced LH release suggesting the initial inhibitory effect *in vivo* is not mediated via a direct effect on the pituitary.

Summary. Time course of 17-β estradiol and luteinizing hormone-releasing hormone effect on LH release was studied using bovine pituitary cells on day 5 of culture. LHRH at concentrations of .1, 1, 10 and 100 ng/ml

increased LH in medium linearly with increasing log concentration of LHRH when present for .75, 1.5, 3, 6 and 24 hr and the percent increase over controls was same at each time period. In addition, estradiol (present for 6, 12 or 24 hr) at .5, 5, and 50 ng/ml also increased LH release linearly both in the presence or absence of LHRH. We conclude that the stimulatory effect of LHRH on LH release from bovine pituitary is consistant over 24 hr and the stimulatory effect of E_2 on both basal and LHRH induced LH release may be mediaed at least in part directly on the pituitary.

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