Changes in the responsiveness of perifused rat adenohypophysial cells to repeated stimulation with luteinizing hormone releasing hormone

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Abstract. The ability of luteinizing hormone releasing hormone (LRH) to stimulate the release of luteinizing hormone (LH) from columns of enzymatically dispersed perifused adenohypophysial cells is being used to study the mechanisms controlling the secretion of LH. LRH stimulated the release in vitro of LH from columns of rat pituitary cells. However, when exposed repeatedly (1 pulse every 12 min) to the same submaximal dose (8 nmol/l) of LRH the cells always exhibited a marked progressive increase and subsequent decrease in their responsiveness. Similar effects occurred when the interval between pulses was extended to 20, 30 or 45 min. The enhanced responsiveness of the cells was prevented by the inclusion of protein synthesis inhibitors, cycloheximide or puromycin, in the perfusion fluid. Cells removed from rats ovariectomized 14 days previously also failed to exhibit increased responsiveness when stimulated repeatedly with LRH. LH secretion was also elicited by K+ (50 nmol/l), 8-bromoadenosine 3'-5'-cyclic monophosphate (8-Br-cAMP, 6 nmol/l), 8-bromoguanosine 3'-5'-cyclic monophosphate (8-Br-cGMP, 6 nmol/l) and a calcium ionophore (A23187, 40 μmol/l) but the responses to these secretagogues differed markedly from those to LRH for the tachyphylaxis which resulted from repeated exposure was not preceded by an increase in responsiveness. The decreased responsiveness to K+ developed in parallel with that to LRH. Diminished responses to the cyclic nucleotides and the Ca2+ ionophore developed more rapidly, but the refractory cells responded readily to stimulation with LRH or K+. The results suggest that the increased responsiveness of the perifused pituitary cells induced by LRH is associated with steroid-dependent protein synthesis and that the secretion of LH elicited by its releasing hormone does not involve cAMP or cGMP.

Many in vitro systems involving static incubation or perfusion of pituitary segments or of acutely dispersed or cultured adenohypophysial cells (Edwardson & Gilbert 1976; Evans et al. 1984; Smith & Vale 1981) have been used to study the functional activity of the gonadotrophs. Dispersed, perifused cells have been employed both for the quantitative determination of LRH and for studies on its mode of action. However, their use has been criticized because some workers have demonstrated persistent changes in the sensitivity of the cells to LRH and its analogues (Yeo et al. 1981), and others have failed to show the self-priming effect of the releasing hormone (Speight & Fink 1981; Loughlin et al. 1981). The experiments described here were done to examine the responsiveness of cells in this system to repeated stimulation with LRH in an attempt to explain some of these discrepancies and to provide further information about the mechanisms which control the secretory activity of the gonadotrophs.

Materials and Methods

Animals

Female Sprague-Dawley rats were obtained from the colony at the Royal Free Hospital School of Medicine and housed, after weaning, 5–6 per cage in a room maintained at 21–23°C with controlled lighting (lights on 07.00–19.00 h). Food and water were available ad libitum. Pituitary tissue was obtained from immature animals (approximately 100 g body weight) and mature...
Preparation of pituitary cell columns

Columns of dispersed pituitary cells were prepared using a modification of the method described by Yeo et al. (1979). The cells of adenohypophyses, removed from rats immediately after decapitation, were dispersed enzymatically with collagenase (Boehringer Mannheim, FRG, 0.1% in Earle's balanced salts medium, pH 7.4, Flow Laboratories, Rickmansworth, England), suspended in an inert matrix (Biogel P2, 0.5 g preswollen), packed into a column which was maintained at 37°C and superfused continuously (0.7 ml/min) with freshly oxygenated Earle's balanced salts medium pH 7.4 containing 0.25% bovine serum albumin (Sigma London Chemical Company Ltd, Fraction 5). After an initial equilibration period of 2.5 h the system was arranged, unless otherwise stated, so that the cells received a 1 min pulse of the test substance every 20 min. The eluates from the column were collected every 2 min and stored at −20°C to await LH assay. Each column contained 6–9 × 10⁶ cells. The viability of the cells was verified by the trypan blue exclusion test. At the end of each experiment the contents of the column were homogenized in 2 ml ice-cold 0.01 M HCl and centrifuged at 2000 × g for 3 min. The supernatant fluid was diluted 1:100 in 0.05 M phosphate buffer pH 7.4 for LH radioimmunoassay.

Determination of LH

LH was measured in duplicate by double antibody radioimmunoassay using reagents supplied by NIADDK and LH-RP-1 (Figs. 2, 3 and 4) and LH-RP-2 (Figs. 1, 5, 6 and 7) as the standard preparations. LH-RP-2 is 61 times more potent than LH-RP-1. At a concentration of 4 ng/ml LH the inter-assay and intra-assay variations were 10.7% and 9%, respectively.

Ovariectomy

Rats weighing between 150–160 g were ovariectomized bilaterally, by the dorsal approach, under ether anaes-
These rats were decapitated 14 days later. Pituitary glands from intact or sham-operated rats from the same batch of animals were used for the control columns.

**Drugs**

Cycloheximide, puromycin, 8-bromoadenosine 3'5'-cyclic monophosphate (8-Br-cAMP), 8-bromoguanosine 3'5'-cyclic monophosphate (8-Br-cGMP) and the calcium ionophore (A23187), were obtained from Sigma London Chemical Co Ltd, England. Potassium chloride was obtained from BDH Chemicals Ltd, Poole, England and LRH from Ayerst Laboratories Ltd, Andover, England. All test substances were dissolved in Earle's balanced salts medium immediately before use and the pH adjusted to 7.4. A23187 was stored as 10 mmol/l in dimethyl sulphoxide (BDH Chemicals Ltd, Poole, England).

**Statistical analysis**

The results were analysed using the paired t-test, the Mann Whitney test (two-tailed) or the Wilcoxon test.

**Results**

Columns of dispersed pituitary cells derived from immature female rats released LH when stimulated repeatedly with pulses of a submaximal dose (Buckingham & Cover 1983) of LRH (8 nmol/l). The increases in the concentration of LH in the eluates were evident first and maximal normally in the fractions collected 4–6 min after the application of each pulse. The LH concentrations always returned to their base line values within 14–16 min, before the initiation of a subsequent response as shown in Fig. 1. Subsequent figures illustrate absolute data from typical experiments, each of which was repeated at least 5 times, and also show the combined results, expressed as percentages of the initial response to LRH, of experiments done on different days. The total amount of LH secreted in response to each stimulus was assessed by determining the area under the curve above the base-line.

LH released from a column of dispersed pituitary cells from immature rats in response to repeated 1 min pulses of LRH (8 nmol/l) given at intervals of (a) 12 min, (b) 30 min and (c) 45 min. The figure shows data from typical experiments.
Effects of cycloheximide on the secretion of LH by columns of dispersed pituitary cells from immature rats which occurs in response to repeated pulses (1/20 min) of LRH (8 nmol/l). ■ = cycloheximide. □ = control. (a) Data from one typical experiment. (b) Pooled data from 6 experiments. Each column represents the mean and is shown with its standard error. • $P < 0.05$ vs control (Wilcoxon test).

The pituitary cells always exhibited an initial progressive increase in their responsiveness when challenged repeatedly (1 pulse every 20 min) with a submaximal dose of LRH (8 nmol/l). Maximal sensitivity occurred with the 6th and 7th pulses, when there was a 2-fold increase in the responsiveness. Further stimulation resulted in diminished responsiveness. The rate of development of tachyphylaxis varied between experiments; in some (e.g. Fig. 2a) it was apparent by the 14th pulse whereas in others (e.g. Fig. 6a) it developed more slowly. In prolonged experiments, in which responses up to 22 pulses of LRH were studied, the reduction in the responsiveness was always significant ($P < 0.05$, Mann Whitney test, n = 5) by the 17th pulse. Similar changes in responsiveness were evident when LRH pulses were given at 12, 30 and 45 min intervals (Fig. 3) or when the pre-incubation period was extended to 6 h (data not shown). The LH contents of the cells in each column at the end of the experiments were not significantly ($P > 0.6$, paired $t$-test, n = 5) different from those at the beginning (105 ± 15%).

The increase in responsiveness was prevented by the inclusion of cycloheximide (36 µmol/l) in the perfusion fluid during the pre-incubation and stimulation periods (Fig. 4). In two experiments, in which 20 successive pulses of the releasing hormone were applied, the cycloheximide treated cells, like the controls, exhibited marked tachyphylaxis, and the responses to the final pulse were approximately 53% of those to the first. Puromycin (114 µmol/l), which was used in place
of cycloheximide in two experiments, had the same effect (results not shown).

Columns of cells prepared from mature rats at random stages of the oestrous cycle exhibited, like those from immature animals, an initial increase and subsequent decrease in their secretory activity when stimulated repeatedly with LRH (8 nmol/l). The initial responses of cells removed from age-matched rats ovariectomized 14 days previously were significantly ($P < 0.05$, paired $t$-test, $n = 5$) greater than those of the intact and sham-opera-

ated controls. However, their responses to the second stimulus were attenuated markedly. The magnitude of the responses diminished gradually with successive stimulations, and the responses to the 16th pulse were only $27 \pm 3.8\%$ of those to the first. These results are shown in Fig. 5.

Fig. 6.
LH released from columns of dispersed pituitary cells in response to repeated pulses (1/20 min) of LRH (8 nmol/l; □) or K$^+$ (50 nmol/l; ■). (a) and (b) show data from two typical experiments. The results from comparable experiments were similar.

Effects of repeated pulses (1/20 min) of (a) 8-Br-cAMP (6 nmol/l), (b) 8-Br-cGMP (6 nmol/l) and (c) the calcium ionophore, A23187 (40 µmol/l) on the release of LH from dispersed pituitary cells. Pulses of LH (8 nmol/l) or K$^+$ (50 nmol/l) were given as indicated. Each profile shows the results from a typical experiment. Control columns prepared from the same batches of cells and run in parallel exhibited the normal pattern of responses to LRH. Data from several comparable experiments were similar.
K+ (50 nmol/l) also stimulated the release of LH from dispersed cells removed from immature animals. The responses to K+ were always reduced with successive stimulations, and tachyphylaxis of the cells to the cation developed in parallel with that to LRH (Fig. 6). The release of LH was also elicited by 8-Br-cAMP (6 nmol/l), but pituitary cells exposed repeatedly to this secretagogue developed tachyphylaxis rapidly (Fig. 7a). Only small, inconsistent responses were provoked by stimulation with either 8-Br-cGMP (6 nmol/l, Fig. 7b) or the calcium ionophore, A23187 (40 μmol/l, Fig. 7c), but cells challenged repeatedly with the cyclic nucleotides or A23187 responded readily to subsequent stimulation with either LRH (8 nmol/l) or K+ (50 nmol/l). Control columns prepared from the same batches of cells and run in parallel, exhibited the normal pattern of enhanced and diminished responsiveness when challenged at 20 min intervals with the releasing hormone.

Discussion

The progressive increase and subsequent decrease in the sensitivity of acutely dispersed perfused adenohypophysial cells to LRH are in accord with our previous observations (Buckingham & Cover 1983). The increased responsiveness of the gonadotrophs to the releasing hormone, frequently called the priming effect, has been demonstrated in vivo (Aiyer et al. 1974) and in vitro using static incubations of pituitary fragments (de Koning et al. 1977) or perfused cultured cells (Smith & Vale 1981). However, relatively few workers (Evans et al. 1984) in the field have been able to demonstrate the priming phenomenon in acutely dispersed perfused cells. The failure of some to show it may have been due to the hormonal state of their experimental animals for, in other in vitro systems, the priming effect is most obvious in tissues removed from animals in the second day of dioestrus or pro-oestrus (Evans et al. 1984). The effect may also have been obscured by experimental design involving, for example, the application of the releasing hormone either in too few consecutive pulses of the same dose or in graded doses administered in ascending order (Speight & Fink 1981).

Although the interval between the pulses of LRH has been shown to influence the sensitivity of the gonadotrophs in vivo (Knobil 1980), variations in pulse frequency did not modify the changes in sensitivity which occurred in our in vitro system. The initial increase in sensitivity appeared, like that seen in other in vitro preparations (de Koning et al. 1977), to be dependent on de novo protein synthesis for it was abolished by protein synthesis inhibitors. It was also associated with the ovarian hormonal status of the animals from which the tissue had been derived. Although the initial response of cells from ovariectomized animals to LRH was considerably greater than that of cells from intact or sham-operated controls, possibly because of the increase in the readily releasable pool of LH caused by gonadectomy (Koiter et al. 1982), the tissue did not exhibit any further increase in sensitivity when challenged repeatedly with the releasing hormone but developed tachyphylaxis rapidly. Studies in other in vitro systems have shown that the priming effect is most obvious in tissues from animals with high circulating levels of oestrogen, e.g. during dioestrus 2 or pro-oestrus (Evans et al. 1984), and preliminary work in this laboratory has produced similar findings with dispersed perfused cells. The present data add further support to the concept that increased responsiveness of the gonadotrophs to LRH, a phenomenon which appears to be important in the sequence of events initiating the LH surge (Aiyer et al. 1974), may be due to oestrogen-induced protein synthesis.

The mechanisms responsible for the development of tachyphylaxis are not clear, but our data indicate that they are independent of changes in the pulse frequency, de novo protein synthesis and the ovarian hormonal status. The tachyphylaxis is not associated with a reduction in the total LH content of the cells. It may reflect changes in receptor number or affinity although Smith et al. (1983) found in their in vitro system that changes in sensitivity were not correlated with changes in LRH binding. Alternatively, it could be due to failure of the tissue to transfer LH from its non-releasable pool to the readily releasable pool. This concept is supported by the finding that the cells stimulated repeatedly with K+, which initiates the secretion of readily releasable hormone only, develop tachyphylaxis to the cation in parallel with that to LRH.

The cyclic nucleotides and Ca++ have all been
implicated as intracellular second messengers for LH (Davis & Hymer 1975; Nakano et al. 1978; Conn et al. 1981). However, our results, like those of Lui & Jackson (1981), do not support the involvement of the nucleotides for, although 8-Br-cAMP and 8-Br-cGMP evoke small increases in LH release, they do not mimic the changes in the secretory activity produced normally by LRH. Furthermore, cells rendered refractory to their effects still respond normally to the releasing hormone and to K+. The weak activity of the calcium ionophore (A23187) was surprising since Smith & Vale (1981) found it to be an effective stimulus of LH release from cultured cells. However, it has been suggested that repeated exposure to A23187 may result in disruption of cellular integrity (Young et al. 1985). Further studies involving the mobilization of extracellular and intracellular Ca++ are essential.

The marked variation in the responsiveness of perfused pituitary cells to LRH renders the preparation totally unsuitable for the biological assay of the releasing hormone. However, the system provides a useful model for studying the mechanisms by which LRH and drugs which mimic or modify its action regulate the secretory activity of the gonadotrophs.

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References


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