Gonadotrophin release by gonadotrophs incubated with gonadotrophin-releasing hormone is independent of intracellular cAMP accumulation

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Abstract. Rat pituitary cells were dispersed with trypsin and separated by sedimentation at unit gravity. The distributions of prolactin (Prl), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were determined, and two enriched cell populations (mammothrophs and gonadotrophs) were subsequently cultured. During a 4 h incubation, gonadotrophin-releasing hormone (GnRH) stimulated the release of LH and of FSH by both the unfractionated population and the enriched gonadotrophs; the magnitude of this stimulation increased with the length of the pre-culture periods, and the amount of LH released into the medium correlated strongly with the amount of FSH, whatever the length of the pre-culture period. The cellular cAMP content was also enhanced during the 4 h incubation, but no correlation was found between the hormone release and the cAMP accumulation. Furthermore, during the first 30 min of incubation with GnRH there was no increase of cellular cAMP, whatever cell population used.

We conclude that the gonadotrophin release was independent of the cAMP accumulation observed in pituitary cells several hours after stimulation by GnRH; consequently, the late increase in the nucleotide is suggested to be a non-specific secondary process.

Since early reports that cyclic adenosine monophosphate (cAMP) may help to regulate the release of gonadotrophins by the pituitary gland (e.g. Jutisz & de la Llosa 1969), the subject has been controversial. Indeed, some studies have revealed a late accumulation of cAMP in pituitary tissue stimulated in vitro by gonadotrophin-releasing hormone (GnRH) or by some of its analogues (Borgeat et al. 1972, 1974; Kaneko et al. 1973; Lippman 1975); furthermore, GnRH has been shown to stimulate the activity of adenylate cyclase in the pituitary (Makino 1973), and cAMP derivatives have been reported to induce the release of gonadotrophin (Ratner 1970; Jutisz & de la Llosa 1970), or, more precisely, the first step ('sensitivity response') of the release (Kercret et al. 1977). However, other groups have thought that this cyclic nucleotide does not play such a role as second messenger, since it does not promote any in vitro increase in the basal releases of the two gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Tang & Spies 1976; Wakahayashi et al. 1973), and since GnRH does not stimulate the adenylate cyclase activity of the pituitary plasma membrane (Théoleyre et al. 1976; Clayton et al. 1978). Recently, Conn et al. (1979) have supported the argument that LH release is independent of cAMP accumulation in cultured rat pituitary cells.

A frequently suggested interpretation of the conflicting findings is that GnRH activates the adenylate cyclase in the gonadotrophs but that the increased level of the nucleotide may be masked by the background level of cAMP in the other cell types of the pituitary gland. In order to test this hypothesis, we have analyzed correlations between...
the GnRH-promoted release of the two gonadotrophins and the cAMP content in an unfractionated cell population and in a gonadotroph-rich cell population. We have also looked for any early rise of cAMP in GnRH-stimulated gonadotrophs and in a cell population containing mainly mammatrophs as a control. Preliminary results have been reported elsewhere (Duval et al. 1980).

Materials and Methods

Male Wistar rats 38 to 44 days old (C. E. R. J., Le Genest) were used. Synthetic GnRH was purchased from Interchim (Montluçon).

Cell dispersion

The cell suspension was prepared essentially as described by Hymer et al. (1973). Briefly, anterior lobes of pituitaries were finely minced and washed in minimum essential medium (Eagle) without glutamine or bicarbonate (SMEM; Gibco biocult). The fragments were transferred to a spinner flask and dispersed enzymatically for 2 h at 37°C in a 10 ml solution of 0.1% trypsin (1:250, Gibco) in SMEM containing 0.1% bovine serum albumin (BSA, Sigma).

After filtration through two layers of gauze to eliminate undigested fragments, the cells were collected by centrifugation and re-suspended in sterile TC medium 199 (Difco) buffered with 25 mM HEPES and containing 0.1% BSA and an antibiotic-antimycotic solution (Penicillin - Streptomycin - Fungizone, Gibco). Using this technique, we regularly recovered 1.2 to 1.4 × 10^6 cells per gland, and the membrane integrity was always 95 to 98% as measured by trypan blue exclusion.

In the first group of experiments, the cells were used either immediately or after an overnight or 3 day pre-culture in the culture medium, which was complete TC medium 199 supplemented with 10% horse serum and 2.5% foetal calf serum (pre-treated with Dextran-coated charcoal so as to eliminate endogenous steroids). In the second group of experiments, the cells were separated as described below.

Cell separation

Following dispersion, the cells were separated at room temperature exactly as described by Hymer et al. (1973), using a 1 × 10^6 600 ml sedimentation chamber. Fractions (30 ml each) were collected (routinely 18 to 19 per run). The cells were pelleted, re-suspended in 1 ml of complete TC medium 199, and counted. In preliminary experiments, aliquots were saved to determine the LH, FSH and Prl content of each fraction and thus to estimate the reproducibility of the method. The enriched cell populations were defined as follows: the 'Prl' population, as fractions 4 to 10 (4 to 7 in the kinetic study), and the

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Separation of male rat anterior pituitary cells by sedimentation at unit gravity. A, Distribution per fractions: ●—● cells; ○—○, Prl; △—△, LH; ▲—▲, FSH. B, Hormone content per cell; symbols are the same as in A. The arrows show the mean amounts per cell of Prl, of LH and of FSH in the unfractionated population.

Fig. 1.

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Basal gonadotrophin release and cellular cAMP content from cultured pituitary cells. Cell suspensions (0 and 16 h pre-culture) or plated cells (3 days pre-culture) were incubated in the absence of GnRH during 4 h in M 199 without serum. Values are expressed as μg LH-RP1, μg FSH-RP1 and pmoles cAMP per 10^6 cells distributed per dish after trypsinization; results are given as mean ± standard deviation; number of determinations between parentheses; each determination for 'GONA' and 'Prl' comes from distinct partition runs, which explains some high standard deviations.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pre-culture time</th>
<th>Medium LH (pg/ml)</th>
<th>Medium FSH (pg/ml)</th>
<th>Cellular cAMP (pmoles/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>0 h</td>
<td>5.11 ± 2.08 (25)</td>
<td>5.28 ± 1.60 (25)</td>
<td>1.21 ± 0.44 (15)</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>2.52 ± 0.96 (24)</td>
<td>2.49 ± 0.61 (24)</td>
<td>0.88 ± 0.30 (20)</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>0.17 ± 0.10 (23)</td>
<td>0.22 ± 0.11 (23)</td>
<td>0.38 ± 0.08 (23)</td>
</tr>
<tr>
<td>'GONA' population</td>
<td>0 h*</td>
<td>21.40 ± 12.36 (6)</td>
<td>18.21 ± 11.04 (6)</td>
<td>3.44 ± 2.60 (6)</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>42.55 ± 47.40 (6)</td>
<td>29.93 ± 15.25 (6)</td>
<td>2.86 ± 1.55 (6)</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>4.65 ± 3.40 (8)</td>
<td>3.22 ± 2.09 (8)</td>
<td>2.03 ± 1.38 (7)</td>
</tr>
<tr>
<td>'Prl' population</td>
<td>0 h*</td>
<td>0.48 ± 0.28 (5)</td>
<td>0.79 ± 0.78 (5)</td>
<td>0.93 ± 0.53 (5)</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>0.31 ± 0.21 (6)</td>
<td>0.31 ± 0.09 (6)</td>
<td>0.43 ± 0.11 (6)</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>0.03 ± 0.03 (8)</td>
<td>0.07 ± 0.05 (8)</td>
<td>0.31 ± 0.11 (7)</td>
</tr>
</tbody>
</table>

* These cells were incubated only 1 h instead of 4 h.

'GONA', or gonadotrophin population, as fractions 11 and beyond (see Results); they were used either immediately or after pre-culture (overnight or for 3 days).

Cell stimulation

In the first group of experiments (with the unfractionated cell population), the cells were distributed into 10 Petri dishes (3.5 to 4.0 × 10^6 cells per dish in 5 ml of TC medium 199) so as to constitute a 5 pair assay, one member of each pair being used as a control (no GnRH) and the other as the experimental (100 ng of GnRH added) sample. The experiment was performed with five independent batches of dispersed cells. In the second group of experiments, each enriched fraction ('Prl' and 'GONA') was distributed into two dishes (one pair of dishes for each fraction); six to eight independent experiments were performed for each time of pre-culture (18 h with cell suspensions and 3 days with plated cells).

Hormone assays and cellular cAMP content

Following the separation at 1 × g, aliquots of the cell suspensions were placed in cold 10 mM NaOH, frozen and thawed, and then centrifuged, and the LH, FSH, and Prl contents of the supernatants were analyzed. After the stimulation with GnRH, the incubation media were spun down to eliminate particles and then frozen until the hormone assays. The hormone contents were estimated by radioimmunoassay using the rat LH, FSH, and Prl kits kindly supplied by Dr. A. F. Parlow and the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD, rat-hormone distribution program).

The assays were performed in the Centre de Radioimmunologie de l'Université de Rennes; polyethylene glycol 6000 was used as a precipitating agent for the gonadotrophins (Kercet & Duval 1976). The cellular cAMP content was determined according to the method of Rosselin & Freychet (1973), with radioimmunoassay performed either as described in the Becton Dickinson protocol or after succinylation of the nucleotide so as to increase the sensitivity of the assay (Delaage et al. 1978).

Statistics

For a given experiment, all the pairs were analyzed in the same radioimmunoassay to eliminate inter-assay variations, but different assays were performed for the different experiments; therefore, the one-sided non-parametric Wilcoxon's signed rank test was preferred to analyze the effects of GnRH. Correlation coefficients between ΔLH, ΔFSH, and ΔcAMP were calculated using each corresponding pair (Snedecor & Cochran 1971).

Results

As shown in Fig. 1A, the cells were distributed throughout the gradient, with the largest number in fraction 5. Prl was found mainly in fractions 4 to 10, and the gonadotrophins, in fractions 8 to 19 (though the LH and FSH profiles were not strictly superimposed). Thus 95% of the mammotrophs...
was found in the 'Prl' population, contaminated with less than 10% of the gonadotrophs; conversely, 90% of the gonadotrophs were pooled in the 'GONA' population, contaminated with less than 5% of the mammotrophs. Though the absolute enrichment of each fraction was not checked by immunocytoLOGY, the gonadotrophin and Prl contents per cell (Fig. 1B) and the basal release of LH and FSH by the populations thus defined (Table 1) confirms the efficiency of the separation process.

The basal release of both the gonadotrophins was very high immediately after cell dispersion and decreased with increasing pre-culture time (Table 1). The data show that there was more cAMP in the 'GONA' population than in the 'Prl' population.

However long the pre-culture period, GnRH stimulated the release of LH and FSH from the unfractionated cells (Fig. 2A), and the cell responsiveness increased with time. No significant increase in the cAMP content was detected imme-

\[ \text{Fig. 2.} \]

Mean increases of LH (ΔLH), FSH (ΔFSH), and cAMP (ΔcAMP) promoted by GnRH for each assay pair in the various cell populations. Open columns, LH; dotted columns, FSH; black columns, cAMP. A, unfractionated cells; B, 'Prl' and 'GONA' populations. The ordinates are given in μg of LH-RP1 and FSH-RP1 per 10⁶ cells and in pmoles of cAMP per 10⁶ cells (note the difference in scale for the 'GONA' population). The corresponding basal releases are given in Table 1. The number of individual experiments is indicated above each column or each group of columns.

The non-parametric Wilcoxon's signed rank test was used; * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \).
Correlations between ΔLH and ΔFSH and between ΔLH and ΔcAMP for each assay pair from the unfractionated cell population. The three pre-culture times have been distinguished for comparison.

Correlations between ΔLH and ΔFSH and between ΔLH and ΔcAMP for each assay pair from the unfractionated cell population. The three pre-culture times have been distinguished for comparison.

diately after dispersion, but the difference became evident after a 16 h pre-culture period. In all cases, the GnRH-stimulated releases of LH and FSH were correlated (Fig. 3), whereas the LH release and the increase in cAMP content were not.

The hypothalamic releasing hormone promoted the release of both gonadotrophins by the cells in the ‘GONA’ population (Fig. 2B). The promoted LH and FSH releases were still highly correlated (r = 0.957, Fig. 4). The increase in cAMP content became significant after the 3rd day of pre-culture, but still no correlation with the release of gonadotrophin was observed (Fig. 4). In the ‘Prl’ population, GnRH not only stimulated the release of LH and FSH because of the slight contamination by gonadotrophs (0.80 µg of LH released/10⁶ cells of the ‘Prl’ population as against 34.64 µg of LH/10⁶ cells of the ‘GONA’ population after 3 days of pre-culture) but also promoted a significant increase in the cAMP content (0.14 pmoles/10⁶ cells, Fig. 2B).

In cells pre-cultured for 3 days, the LH release started soon after GnRH stimulation and proceeded quite linearly, both in the whole cell population and in the ‘GONA’ population (Fig. 5). Yet in no case did we find evidence of cAMP increase in any cell population from zero time to 30 min of stimulation.

Discussion

In our hands, trypsin appears far better than collagenase for the dispersion of pituitary cells, since it gave higher yields and more viable cells. Freshly dispersed cells had a high basal release of gonadotrophins and were rather unresponsive to GnRH.
Some plasma membrane components were probably damaged by the enzymatic treatment, but our cells seemed to recover faster than those of Denef et al. (1978): in our experiments the stimulation by GnRH was already great after one night of preculture, as was also found by Naor et al. (1978). But, in agreement with Denef's group, we have found (data not shown) a 3 day pre-culture time necessary to restore a good efficiency (half maximal stimulation at about $5 \times 10^{-9}$ M GnRH). Unlike Vale et al. (1972), we found that the GnRH-promoted releases of the two gonadotrophins were highly correlated however long the pre-culture; this observation supports the hypothesis of a single trigger mechanism for LH and FSH, the efficiency of which is restored once the cellular receptors for GnRH are repaired.

Our results with the unfractionated cell population agree with the recent findings of Conn et al. (1979) and of Bérault et al. (1980); i.e., no correlation was found between hormone release and cellular cAMP content. Prior cell fractionation turned to be necessary before definite conclusions could be drawn, since the gonadotrophs represent a small fraction of the pituitary cells. We used the currently most convenient way of obtaining enriched populations, sedimentation at unit gravity, as initially described by Hymer et al. (1973). Using radioimmunoassays, we analyzed the distributions of Prl, LH and FSH precisely in order to obtain not only a

\[
\begin{align*}
\Delta LH & \quad r = .957 \\
\Delta FSH & \\
\Delta LH & \quad r = -.347 \\
\Delta cAMP & 
\end{align*}
\]

**Fig. 4.**

Correlation between $\Delta LH$ and $\Delta FSH$ and between $\Delta LH$ and $\Delta cAMP$ for each assay pair from the 'GONA' population. Two pre-culture times were used and are distinguished on the figure (○—○, 16 h, and ▲—▲, 3 days) but their data were pooled for calculation of the correlation coefficient.
Stimulation of (A) the unfractionated cells, (B) the 'GONA' population (gonadotroph-enriched), and (C) the Prl population (mammotroph-enriched) by GnRH. •—•, LH released per 10⁶ cells; o—o, cellular cAMP content per 10⁶ cells. Each point is the mean of 4 determinations (mean ± standard deviation). For the 'Prl' population, all the LH values fall below the limit of detection (< 0.03 μg LH per 10⁶ cells).

GnRH stimulated the releases of both the gonadotrophins from the 'GONA' fraction, and these releases were still highly correlated after 4 h of incubation; although the cAMP also increased in that period, it was still not correlated with the hormone release. In the 'Prl' population, a rise in the cellular cAMP content was also promoted during the 4 h incubation (+ 0.14 pmoles/10⁶ cells); this represents a mean 45% increase over the basal cellular content (0.31 pmoles/10⁶ cells) and was of the same order of magnitude as the 57% increase (+ 1.16 pmoles/10⁶ cells) over the basal content (2.03 pmoles/10⁶ cells) observed in the 'GONA' fraction. Consequently, this GnRH-stimulated rise can hardly be assigned to the slight contamination...
of the mammotrophs by the few small gonadotrophs. Moreover, the results of the kinetic study show for the first time that GnRH did not promote any early increase of cAMP in the gonadotrophs.

All these data constitute more evidence in favour of the hypothesis that gonadotrophin release is independent of a previous rise in cellular cAMP. In explanation of the late cAMP increase in the various cell populations, it can be suggested that receptors for GnRH exist on the plasma membranes of several cell types, including mammotrophs, and that adenylate cyclase can be activated in any cell by this peptide (though recent results show that, in vivo, GnRH binds to purified plasma membranes without activating the cyclase (Théoleyre et al. 1976; Clayton et al. 1978)). Alternatively, and more probably, the late rise in cAMP may be a secondary phenomenon promoted by some metabolite produced in the medium during the 4 h incubation.

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References


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