ACUTE INFLUENCE OF LH AND FSH ON CYCLIC AMP FORMATION IN ISOLATED GRANULOSA CELLS OF THE RAT

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ABSTRACT

A technique for the mechanical isolation of granulosa cells from the rat ovary is described. Cyclic AMP formation by the isolated granulosa cells of the follicles in various stages of development was studied in response to the administration in vitro of gonadotrophins. In granulosa cells from small to medium-sized follicles FSH but not LH stimulated cAMP formation, while in cells from pre-ovulatory follicles both gonadotrophins had a stimulatory effect. The effects of both gonadotrophins were transient with a maximal response after 15 to 60 min of incubation. In the presence of the phosphodiesterase inhibitor, 3-isobutyl-methylxanthine, the action of FSH was potentiated and prolonged while the response to LH was unaffected. These data indicate that both gonadotrophins activate the adenylate cyclase system of the isolated granulosa cells while FSH in addition stimulates the phosphodiesterase activity.

Consecutive determinations of cAMP during and after the pre-ovulatory LH-FSH surge, demonstrated a rise of cAMP levels in granulosa cells from the pre-ovulatory follicles following endogenous gonadotrophin release. cAMP levels remained high or increased until the time of ovulation.

In experiments on isolated granulosa cells from porcine ovaries (Kolena & Channing 1971, 1972), it was demonstrated that the addition of either LH or FSH caused an acute stimulation of the formation of cAMP in these cells. The stimulatory effect of LH was greater than that of FSH. Submaximal concentrations of the two gonadotrophins were additive while maximal concentrations
were not. Addition of the phosphodiesterase (PDE) inhibitor, aminophylline, augmented the effects of gonadotrophins on cAMP formation (Kolena & Channing 1972) while aminophylline alone had no effect. In a subsequent study by the same group (Channing 1973, 1974), the size of the follicles from which the granulosa cells were isolated was investigated. It was found that cells from small and medium-sized follicles contained less cAMP when compared to cells isolated from large follicles. Furthermore, the stimulatory effect of LH was most pronounced in cells from large follicles. The increased sensitivity to LH has been ascribed to the development of LH receptors on the surface of the granulosa cells (Rajaniemi & Vanha-Perttula 1972; Nimrod et al. 1976; Zeleznik et al. 1974; Lee 1976).

The present study describes a new technique for the mechanical isolation of morphologically and functionally well-preserved granulosa cells. Using this cell suspension, experiments were undertaken to analyse further the influence of LH and FSH on cAMP formation in granulosa cells isolated from rat ovarian follicles at various stages of development. The rat model was chosen because it allows a more exact control of the endocrine situation of the donor animal as compared to many other species.

**Material and Methods**

Sprague-Dawley rats obtained from Anticimex Ltd., Sweden were kept in rooms with constant temperature (24–26°C) and humidity (50–55%o). They were exposed to 14 h of controlled illumination starting at 6 a.m. The rats were fed a standardized pellet diet and water *ad libitum*. Twenty h prior to the start of the experiments the animals were deprived of food.

Isolated granulosa cells were obtained either from ovaries of 25-day old immature rats or from 32-day old rats pre-treated with a single subcutaneous injection of PMSG (10 IU) in the morning (8 a.m.) of day 30. This treatment leads to the development of 13–17 pre-ovulatory follicles/rat, and an endogenous LH peak between 5–8 p.m. on day 32, with ovulation occurring in the early morning of day 33 (for details see Fuxe et al. 1972; Hillensjö et al. 1974).

**Isolation procedure**

The rats were killed by cervical fracture and the ovaries immediately removed and placed on ice in a Krebs bicarbonate buffer (see composition below). Using a stereo-microscope, the ovaries were trimmed of extraneous tissues and bursae. The ovaries were transferred to a hollow glass cup containing chilled buffer without substrate. The follicles were punctured by means of stainless steel needles in order to release the granulosa cells into the medium. In ovaries from the 25-day old rats, the larger follicles (diameter varying between 100–400 μm) were punctured, while in those from 32-day old rats only the pre-ovulatory follicles (diameter approximately 600 μm) were punctured (Fig. 1). The theca capsules and other ovarian tissues were removed.

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**Fig. 1.**

Schematic isolation procedure for granulosa cells from pre-ovulatory rat follicles.

**Fig. 2.**

Celluscopic analysis of granulosa cells isolated either from small and medium-sized follicles from ovaries of 25-day old untreated rats or from pre-ovulatory follicles from ovaries of PMSG-treated rats. The mean cellular diameter for both types of cells is approximately 9 \( \mu \text{m} \).
and the cell-suspension containing granulosa cells and follicular oocytes was transferred by Pasteur pipettes to glass tubes. These were accelerated in a swing-out rotor for 20 seconds to give a maximal speed corresponding to 900 x g for 5 seconds. After centrifugation, the supernatant was carefully decanted. A loose cell pellet remained at the bottom of the tubes. By use of 14C-labelled sucrose and dry weight determinations it was found that the "extra-cellular" space of this pellet corresponded to 90% of the weight. If the cells were more firmly centrifuged to reduce the amount of "pellet water" the morphology of the granulosa cells was affected and a high percentage of isolated nuclei appeared. The cellular concentration in the final incubation medium varied between \(5 \times 10^5 - 5 \times 10^6\) per ml. The purity of the cell samples was checked by light microscopy and was found to be between 80-90%. A cellscope analysis (Fig. 2) utilizing a Coulter counter ZB channel analyzer showed that the cellular diameter of the majority of the isolated material was approximately 9 \(\mu m\). Smaller particles were mainly isolated whole nuclei, and larger particles consisted of aggregated cells. No significant difference in cell diameter was found between cells isolated from ovaries of 25-day old rats and from those of pre-ovulatory follicles.

In some cases, electronmicroscopical analysis before and after incubation of the cells was kindly performed by Prof. Lars Bjersing, Dept. of Pathology, Umeå University, Sweden. The sections revealed that most of the cells analysed had preserved subcellular formations. The functional capacity of the granulosa cells obtained by this procedure has been tested in short-term incubations by measuring both their ability to form steroids (Hamberger et al. 1978) and to incorporate radio-labelled amino acids into protein (unpublished data) in acute incubations. Both these parameters increase linearly during the 4 h experimental period.

**Incubation and analysis**

The cell suspensions were incubated for various periods in sealed glass tubes that had been aerated with 95% \(O_2\) + 5% \(CO_2\) for 30 seconds prior to the incubations. The gyratory water bath had a constant temperature of 37°C and was fixed at 50 r. p. m. After 5 min of "pre-incubation", the gonadotrophins were added and the tubes aerated once more. In longer incubation experiments the tubes were aerated every second hour.

Following incubation, the cells and the medium were chilled immediately; transferred to glass homogenizers, and homogenized at a final trichloroacetic acid concentration of 5%. Protein was determined according to Lowry et al. (1951) and cAMP according to Gilman (1970). The separation of the cells and the medium after incubation but before the determination of cAMP in a small number of initial experiments (Table 2) was shown to be unreliable, since the separation in itself caused damage of the cells to a certain and not reproducible degree. The samples were stored at -70°C until analysed. cAMP formation has consistently been expressed as pmol/mg protein. Other investigators (e.g. Kolena & Channing 1972) have expressed cAMP formation per million granulosa cells in order to be able to compare cell number and protein content.

In the present study aliquots of granulosa cells from 25- and 32-day old rats were first counted and thereafter taken for protein determinations. It was then found that although the cellular diameters of the two types of granulosa cells were similar (see Fig. 2), the protein content varied. Granulosa cells isolated from ovaries of 25-day old rats have a mean protein content of 0.067 mg per 1 million cells while the corresponding values for granulosa cells isolated from ovaries of 32-day old rats is 0.084 mg. This difference was highly significant \((P < 0.001)\).
Chemicals and hormones

\[^3\text{H}\]Cyclic adenosine-3',5'-monophosphate (specific activity 20 Ci/mmol) was purchased from New England Nuclear Co., Boston, USA. Binding and inhibitory proteins for cAMP assays were extracted from rabbit muscle and the cAMP determinations performed according to Gilman (1970). Gonadotrophic hormones were supplied by the Endocrinology Study Section of the National Institutes of Health. Bovine LH (NIH-LH-B8) ovine FSH (NIH-FSH-S9) and prolactin (NIH-P-S3) were used throughout. The phosphodiesterase (PDE) inhibitor 3-isobutyl methylxanthine (IBMX) was kindly supplied by Searle AG, Chicago, USA. \[^{14}\text{C}\]Sucrose (U) (specific activity 220 mCi/mmol) was purchased from NEN Chemicals GmbH, West Germany.

Incubation medium

Modified Krebs Ringer bicarbonate buffer (1.25 mM CaCl\(_2\)) with or without 5.5 mM glucose (pH = 7.4).

Statistical procedure

The results were analyzed using Student’s t-test (2 groups) or analysis of variance (with one criterion of classification) when more than two groups were compared. A P-value of 0.05 or less was considered significant.

RESULTS

Cyclic AMP formation by granulosa cells from ovaries of 25-day old immature rats

In the first series of experiments illustrated in Fig. 3, isolated granulosa cells were incubated for 60 min in the absence and presence of LH and FSH at various concentrations. FSH increased the amount of cAMP in the medium plus tissue with a maximal stimulatory effect at a hormonal concentration of 1 \(\mu\)g/ml while LH did not affect the amount of cAMP present in medium plus tissue.

In the presence of IBMX (0.2 mM) a significant increase \((P < 0.01)\) of the cAMP levels was registered after 60 min (Table 1) and the stimulatory effect of FSH was also significantly potentiated in the presence of IBMX. LH, however, showed no significant effects on the cAMP levels in the presence of IBMX (Table 1).

Cyclic AMP formation by granulosa cells from pre-ovulatory follicles

In the granulosa cells that were isolated from pre-ovulatory follicles in the morning (10–12 a.m.) of day 32 before the endogenous LH-FSH-peak, prolonged incubation of the cells in the absence of a PDE inhibitor in the medium resulted in a gradual decrease in the amount of cAMP present in medium plus tissue. This decrease was partially inhibited by the addition of the PDE in-
cAMP content (tissue + medium) in granulosa cells isolated from small and medium-sized follicles from 25-day old untreated rats. There are 4–5 observations in each group. The hormones were added after 5 min pre-incubation and the cells incubated for 60 min. The effects of FSH at 1, 10 and 100 µg/ml are significant ($P < 0.01$). Vertical lines above the bars indicate SEM.

Table 1.
cAMP formation by isolated granulosa cells from ovaries of 25-day old rats in the absence and presence of the phosphodiesterase inhibitor IBMX. cAMP was determined in medium plus tissue and expressed as pmol/mg protein.

<table>
<thead>
<tr>
<th>Groups</th>
<th>IBMX (0.2 mM)</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>50.8 ± 13.0 (4)</td>
</tr>
<tr>
<td>LH 0.1 µg/ml</td>
<td>-</td>
<td>35.2 ± 3.2 (4)</td>
</tr>
<tr>
<td>LH 10.0 µg/ml</td>
<td>-</td>
<td>41.4 ± 10.1 (3)</td>
</tr>
<tr>
<td>LH 100.0 µg/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FSH 10.0 µg/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>50.2 ± 3.6 (4)</td>
</tr>
<tr>
<td>LH 10.0 µg/ml</td>
<td>+</td>
<td>39.7 ± 2.4 (3)</td>
</tr>
<tr>
<td>FSH 10.0 µg/ml</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Significant effect above the respective control level $P < 0.01$. 

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Fig. 4.
cAMP content (tissue + medium) in granulosa cells from pre-ovulatory follicles isolated in the morning (10 a.m.–1 p.m.) before the endogenous LH peak. The cells are incubated for various periods in the absence and presence of the PDE inhibitor, IBMX.

Fig. 5.
cAMP content (tissue + medium) in granulosa cells from pre-ovulatory follicles isolated in the morning (10–12 a.m.) before the endogenous LH peak. The hormones were added after a pre-incubation period of 5 min not indicated in the figure. The stimulatory effects of both gonadotrophins at the two concentrations were significant after 15 min incubation ($P < 0.02$). No significant difference from the control level was found after 5 min incubation. SEM values are too small to be indicated in the figure.
hibitor IBMX (Fig. 4). Due to the continued degradation of cAMP in the absence of IBMX, time curves for the effects of the gonadotrophins were calculated as a per cent of the respective controls. Fig. 5 shows the results from experiments where two concentrations (1 and 10 μg/ml) of LH and FSH were used for 5 to 60 min. After 15 min both LH and FSH significantly stimulated cAMP at either of the concentrations used. The effect of FSH (10 μg/ml) was significantly higher than that of LH at the same concentration at both 15 and 60 min. Addition of gonadotrophins (FSH or LH) in higher concentrations (50 or 100 μg/ml) did not cause any further stimulation. However, in certain experiments a reduction of the stimulatory effect was registered when compared to the effect of 10 μg/ml. Prolactin at concentration of 10–50 μg/ml was totally ineffective after incubation both at 15 and 60 min.

A submaximal concentration of either FSH or LH (1 μg/ml) was used in prolonged incubations (up to 4 h). As demonstrated in Fig. 6 the stimulatory effects of both gonadotrophins were maximal at this hormonal concentration after 60 min and vanished after 2 h. In order to analyse further this latter effect, corresponding experiments were performed in the presence of IBMX (Fig. 7) (compare Fig. 4 for control levels). The PDE inhibitor did not influence the stimulatory effect caused by LH after 15 and 60 min and did not prolong the stimulatory effect. The stimulatory effect of FSH, however, was considerably prolonged to at least 4 h.

![Graph](image)

**Fig. 6.** cAMP content (tissue + medium) in granulosa cells from pre-ovulatory follicles isolated in the morning (10 a.m.–1 p.m.) before the endogenous LH peak. The hormones were added after a pre-incubation period of 5 min (not indicated in the figure). There are 4 observations at each point. Vertical bars indicate SEM. No significant stimulation by either hormone was found after 2 and 4 h incubation.
cAMP content (tissue + medium) in granulosa cells from pre-ovulatory follicles isolated in the morning (10–12 a.m.) before the endogenous LH peak. The cells were incubated in the presence of the PDE inhibitor IBMX (0.2 mM). After 2 and 4 h incubation there is a remaining stimulatory effect of FSH but no effect with LH (compare Fig. 6 illustrating corresponding incubations in the absence of IBMX).

In one series of experiments granulosa cells were isolated and taken for immediate determination of cAMP content before, during and after the endogenous LH-FSH peak on day 32. The endogenous LH peak in the PMSG model used occurs between 5 and 8 p.m. (Hillensjö et al. 1974). A small but significant rise in the cAMP levels of granulosa cells was registered when the cells were isolated in the evening following the endogenous LH-FSH peak (Table 2).

**Table 2.**

<table>
<thead>
<tr>
<th>Age of rat</th>
<th>Day 32</th>
<th>Day 33</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8–12 a.m.</td>
<td>2–4 p.m.</td>
</tr>
<tr>
<td>cAMP (pmol/mg protein)</td>
<td>71.34±4.33</td>
<td>66.47±5.89</td>
</tr>
<tr>
<td>No. of observations</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.

Influence of LH (NIH-LH-B6) on the levels of tissue cAMP in isolated granulosa cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Incubation period</th>
<th>Cyclic 3',5' AMP (pmol/mg protein)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>LH 1 μg/ml</td>
</tr>
<tr>
<td>Granulosa cells from small follicles*</td>
<td>15</td>
<td>3.20 ± 0.70</td>
<td>4.30 ± 1.30</td>
</tr>
<tr>
<td>Granulosa cells from pre-ovulatory follicles**</td>
<td>15</td>
<td>7.03 ± 0.35</td>
<td>17.43 ± 1.34</td>
</tr>
</tbody>
</table>

* Follicles isolated from ovaries of 27-day old rats.
** Follicles isolated from ovaries of 32-day old rats injected with 10 IU PMSG at 30 days of age.

Tissue cAMP content in granulosa cells from 25-day old immature rats or from pre-ovulatory follicles

In a small series of experiments cAMP was determined only in the granulosa cell pellet obtained after incubation and centrifugation. The cells were incubated in the absence and the presence of LH (1 μg/ml) for 15 min. As seen from Table 3, the control levels correspond to only 5–10% of the levels obtained when both tissue and medium are determined for cAMP (compare Table 1 and Fig. 5). The lack of responsiveness to LH in granulosa cells from immature ovaries and the stimulatory effect by the gonadotrophic hormone in cells isolated from pre-ovulatory follicles is in accordance with the findings obtained when both medium and tissue cAMP were determined.

DISCUSSION

FSH but not LH stimulated the formation of cAMP in granulosa cells from ovaries of untreated immature rats. This difference in effects has been attributed to the presence of FSH receptors and the lack of LH receptors in granulosa cells mainly found in small and medium-sized follicles (Midgley 1973; Nimrod et al. 1976). In studies on porcine granulosa cells from follicles with a diameter of 3–5 mm (Kolena & Channing 1971, 1972), it was found that both LH and FSH could elevate the cellular levels of cAMP. Whether these granulosa cells represent a more developed stage than granulosa cells from ovaries of 25-day old rats is not known.

In recent studies from this laboratory (Selstam et al. 1976; Selstam & Rosberg 1976a) on whole isolated ovaries from immature rats, an increase in the tissue
content of cAMP was found when either LH or FSH was administered \textit{in vivo} or \textit{in vitro}. The lack of stimulatory effect of LH on the isolated granulosa cells from immature rat ovaries reported in the present study gives indirect evidence for a stimulatory effect of LH in other ovarian cell types. Unpublished preliminary data have also demonstrated a stimulatory effect of LH \textit{in vitro} on cAMP formation by interstitial tissue from immature rat ovaries (Hamberger, to be published). A stimulatory effect of LH on cAMP formation in isolated theca cells is another possibility not yet thoroughly investigated in the rat, but recent publications (Weiss et al. 1976; Weiss & Armstrong 1977) on isolated theca cells from sheep ovaries have demonstrated clear stimulatory effects of LH. FSH and HCG. In other experiments from this laboratory, on whole ovaries of immature rats, Selstam & Rosberg (1976a,b) found a selective influence of FSH on cAMP phosphodiesterase (PDE). The present study on granulosa cells from ovaries of immature rats demonstrates a selective potentiation of the FSH effect in the presence of the PDE inhibitor IBMX.

A lack of effect of LH on PDE activity has been reported earlier in studies on the homogenates of rabbit and bovine ovaries (Dorrington & Kilpatrick 1969; Marsh 1970). In an attempt to elucidate this problem, experiments were performed on granulosa cells isolated from pre-ovulatory rat follicles, obtained by the use of a PMSG model (Fuxe et al. 1972; Hillensjö et al. 1974). Granulosa cells isolated 5–7 h prior to the endogenous LH-FSH peak have a more pronounced PDE activity than granulosa cells from ovaries of immature rats (compare Table 1 and Fig. 4). The addition of 0.2 mM IBMX partially blocked the PDE activity during at least 4 h incubation. In the absence of a PDE inhibitor both LH and FSH had stimulatory effects on cAMP formation although the effect of FSH seemed more pronounced at the maximal concentrations of both gonadotrophins (Fig. 5). Further, the stimulatory effects of FSH and LH were transient and disappeared completely with prolonged incubation (2 and 4 h). When granulosa cells were incubated for 2 and 4 h in the presence of IBMX the stimulatory effect of FSH remained while the effect of LH was unaffected. The present data on isolated granulosa cells from pre-ovulatory follicles support the assumption that both gonadotrophins acutely activate adenylate cyclase. This has been reported in earlier publications on ovarian tissues from other species (Marsh 1970, 1971; Kolen & Channing 1972; Hunzicker-Dunn & Birnbaumer 1976). However, our data suggest that there is a difference in gonadotrophin action which is associated with the degradation of cAMP – \textit{i.e.} that FSH but not LH stimulates the PDE activity.

In an earlier publication from this laboratory, Nilsson et al. (1975) found a small rise in cAMP levels of whole ovaries after the endogenous LH-FSH peak in the afternoon of day 32 in the same PMSG model which we have used in the present study. A small but significant increase of the same magnitude as for the whole ovary was found in granulosa cells isolated after the LH-FSH
peak but unlike the whole isolated ovaries, the isolated granulosa cells showed no decrease in cAMP levels up to approximately 2 h before ovulation (Table 2).

In one series of experiments (Table 3) the cAMP content in granulosa cells isolated from ovaries of 27-day old immature rats and from pre-ovulatory follicles was determined without measuring the amount of cAMP in the incubation medium. This experimental design was used to reproduce the experimental procedure utilized by Kolena & Channing (1971, 1972) and other investigators. The levels of cAMP in rat granulosa cell pellets also correspond very closely to those reported for porcine granulosa cell pellets by Kolena & Channing (1972). The amount of cAMP present in the tissue was only 10% of the total amount of cAMP in both tissue and medium after 15 min incubation in the absence of a PDE inhibitor. In comparing the results of Kolena & Channing (1971, 1972) with those of the present study, it must thus be kept in mind that in the former studies only "tissue cAMP" was determined while in the majority of our own experiments the amount of cAMP in both tissue and medium was determined without preceding separation. This difference might be of special importance when stimulatory effects of gonadotrophins are studied since Selstam et al. (1976) in experiments on whole isolated incubated ovaries from immature rats found that the release of cAMP from tissue to medium was 2–3 times greater in the presence of LH when compared to FSH.

In studies on isolated granulosa cells from sheep follicles (diameter 4–6 mm) Weiss et al. (1976) reported levels of tissue cAMP which were low compared with the cAMP levels in both porcine and rat granulosa cells. This discrepancy might be due partly to species differences but possibly to the isolation procedure. The latter explanation may further be strengthened by the fact that these investigators could not demonstrate any significant stimulation of cAMP formation by gonadotrophins or PGE2.

In conclusion – the capacity of rat granulosa cells to form cAMP is not markedly increased with the development of the follicle. cAMP formation in granulosa cells from small to medium-sized follicles is stimulated by FSH while LH has no effect. The formation of cAMP in granulosa cells from pre-ovulatory follicles can be stimulated by both LH and FSH although the mechanism(s) for the formation and degradation of cAMP in tissue and incubation medium seem(s) to be different.

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