Effect of gonadotrophin-releasing hormone (GnRH) and GnRH agonists upon accumulation of progesterone, cAMP and prostaglandin in isolated preovulatory rat follicles

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Abstract. To study the acute and direct effects of GnRH agonists preovulatory follicles were isolated from PMSG-treated immature rats and incubated for 15–360 min in modified Kreb's bicarbonate buffer. The levels of cAMP, prostaglandin E, and progesterone were analysed in the tissue and/or incubation media. GnRH and two GnRH agonists produced a dose-dependent stimulation of progesterone production with maximal levels 5–6-fold higher than the control group. As compared to LH the magnitude of this effect was small and was detected only after 240–360 min of incubation. GnRH also stimulated prostaglandin E accumulation and this effect was as pronounced as for LH. There were no detectable changes in cAMP levels for any concentration of GnRH when the incubation time varied between 15 and 120 min whether or not a phosphodiesterase inhibitor was present, but after 240 min of incubation a 2-fold increase in cAMP was found. Consistent with previous results, LH caused a pronounced (40–50-fold) increase in follicular cAMP which was already detectable after 15 min of incubation. Indomethacin abolished the rise in prostaglandin E induced either by GnRH or LH but did not affect the response in terms of cAMP or progesterone, and did not affect the stimulation of meiotic maturation of the follicle-enclosed oocytes caused by the hormones. It is concluded that GnRH can exert acute and LH-like stimulatory effects on the preovulatory rat follicle but that the mechanism of GnRH action is different from that of LH.

Chronic treatment with GnRH agonists causes 'paradoxical' anti-fertility actions in humans and experimental animals (Banik & Givner 1975; Nillius et al. 1978). These effects may partly be explained by a hyperstimulation and desensitization of the pituitary gland. GnRH also influences ovarian function in hypophysectomized animals and direct pharmacological actions by GnRH agonists on the gonad are now widely recognized. Specific high-affinity receptors for GnRH in the gonads have been demonstrated (reviewed by Clayton & Catt 1981). Most of the direct effects observed so far are inhibitory in character and include inhibition of ovarian growth (Rippel & Johnson 1976), development of gonadotrophin receptors (Harwood et al. 1980), and steroidogenesis (Hsueh & Erickson 1979; Hsueh et al. 1980). The inhibitory effects of GnRH have been observed for different ovarian compartments including granulosa cells (eg Hsueh & Erickson 1979), luteal cells (Behrman et al. 1980) and interstitial cells (Magoffin et al. 1981). These direct inhibitory actions of GnRH have, with few exceptions, been seen following prolonged treatment with pharmacological doses of GnRH agonists in vivo or in vitro.

In contrast to the above-mentioned inhibitory actions we have observed direct stimulatory effects of GnRH agonists following acute administration. These effects include in vitro stimulation of prostaglandin (Clark et al. 1980) and progesterone
synthesis (Clark & Marsh 1980; Clark 1982) in isolated rat granulosa cells, induction of meiotic maturation of follicle-enclosed rat oocytes, stimulation of oocyte respiration, and of follicular lactate formation in vitro (Hillensjö & LeMaire 1980; Magnusson & LeMaire 1981). Recently it was reported that a single injection of a pharmacological dose of GnRH agonist in rats, which had been hypophysectomized on the morning of pro-oestrus, stimulated resumption of oocyte meiosis, increased plasma progesterone levels and caused ovulation (Ekholm et al. 1981; Corbin & Bex 1981) and furthermore that ovulation (follicular rupture) appeared to be related to the stimulation of ovarian prostaglandin levels (Ekholm et al. 1982). Thus a number of LH-like responses on preovulatory follicular functions have been observed following acute GnRH treatment in vivo and in vitro. An interesting distinction between the response to LH and GnRH in the isolated granulosa cells was the response in terms of cAMP accumulation, which was markedly stimulated by LH whereas no changes in cAMP could be detected following GnRH stimulation (Clark et al. 1980).

The aim of the present work was to study the effect of GnRH agonists upon the accumulation of cAMP, progesterone and prostaglandin in whole isolated follicles obtained from PMSG-treated immature rats.

Materials and Methods

Animals

Immature, 26-days-old Sprague-Dawley rats were injected with 10 IU PMSG to synchronize and stimulate follicular growth (Ekholm et al. 1981). The animals were killed by cervical dislocation on the morning of day 28, before the endogenous LH-surge, and ovaries were excised.

Incubation procedure

Ovaries were cleaned of adherent tissue and preovulatory follicles isolated by dissection under a stereomicroscope as described (Hillensjö 1976). Follicles were incubated in 0.7 ml medium (4–7 follicles per flask) for 15–360 min at 37°C. The medium was a modified Kreb's bicarbonate buffer with 1 mg/ml glucose and 1% BSA and was equilibrated with 95% O₂–5% CO₂. The various test substances were present in the medium from the start. Following the incubation the medium was frozen for later analysis and the follicles either homogenized to determine tissue levels of cAMP or progesterone or opened to recover the oocyte for microscopical examination. Each experiment was repeated at least twice.

Chemicals and hormones

PMSG was obtained from Sigma Chemical Co. (St. Louis, Mo). Ovine LH (NIH-S19) was provided by the Hormone Distribution Program of the NIAMDD. Synthetic GnRH was obtained from Ferring Ltd. or Sigma. D-Ala²,des-gly-NH₂-LRH-ethylamide (GnRHa) was obtained from The Beckman Co., Palo Alto, CA or from Sigma. D-Leu⁶,des-gly-NH₂-LRH-ethylamide (GnRHB) was from Abbot Laboratories. D-pGlu, D-Phe², D-Trp³,LRH (antagonist) was purchased from Boehringer Mannheim Chemical Co., Indianapolis, IND. TRH was from Hoffman-LaRoche, Switzerland. 3-iso-butyl-methyl-xanthine (MIX) was from Aldrich Chemical Co., Belgium. Labelled [1,2,6,7-³H]progesterone was from Amersham Radiochemical Centre and labelled [³H-6]cAMP from New England Nuclear.

Progesterone assay

Progesterone was analyzed by RIA in aliquots of the incubation media and in certain experiments in follicular homogenates using a specific antiserum generated against the 11-hydroxy-progesterone derivative (Lindner & Bauminger 1974) and kindly supplied by Drs. Hans Lindner and Fortuna Kohan, The Weizmann Institute of Science, Israel. The procedure followed earlier reports using the present conditions (Hillensjö et al. 1976) except that the extraction step was omitted when medium levels were assayed, since a good correlation had been found between values obtained with this direct assay as compared to those obtained with the previously used assay which included petroleum-ether extraction (r = 0.98, n = 36).

cAMP assay

cAMP was measured separately in follicular homogenates and incubation media. Follicles were homogenized in 5% TCA, extracted with ether and assayed by a protein binding assay (Gilman 1970) modified as described (Nilsen et al. 1974; Khan et al. 1980). Protein content was measured according to Lowry et al. (1951).

Prostaglandin assay

Samples of incubation media were extracted and assayed for prostaglandin E by RIA as described previously (LeMaire et al. 1973; Clark et al. 1978).

Oocyte meiosis

After recovery from the follicles oocytes were examined with Nomarski interference contrast microscopy to reveal absence or presence of nuclear structures. Oocytes with germinal vesicle breakdown (GVB) were considered as maturing.
Effect of different concentrations of peptides upon progesterone secretion during a 4 h incubation of follicles. The accumulation of steroid in the medium was determined. The data shown are pooled from several different experiments and represent the means ± SE. For TRH the SE is smaller than the size of the symbol. There are 26 observations in the control group (C) and 5–14 observations in each experimental group. For abbreviations see Materials and Methods. The lowest concentrations that gave a significant increase (*P < 0.05 with analysis of variance) compared to the control group were: GnRH, 1 µg/ml; GnRHa and GnRHb 1 ng/ml.

Statistics
Statistical differences among groups were calculated by analysis of variance followed by Student-Newman-Kuel's multiple range test. When there was heterogenous variance logarithmic transformation of the data was made before the statistical procedure was applied. A P-value less than 0.05 was considered significant. The data presented below in the tables and figures are either pooled from several different experiments or represent individual typical experiments.

Results

Progesterone accumulation
The effect of various concentrations of GnRH and the two analogs on progesterone accumulation during 4 h of incubation is depicted in Fig. 1. A 5–6-fold stimulation was found for all these compounds but their relative potencies varied. The lowest concentrations that gave significant stimulation of progesterone accumulation were 1 ng/ml for the analogs and 1 µg/ml for the parent compound. TRH was without effect. In separate ex-

Time-course of progesterone accumulation in the incubation medium in absence and presence of GnRHa (1 µg/ml). The data are pooled from 2–3 experiments and the figure shows the mean ± SE of 8–14 observations. A significant stimulation (*P < 0.01) by GnRHa compared to the control group was found at 4 and 6 h of incubation. After 2 and 4 h of incubation the levels of progesterone in the control group was lower than the practical detection limit (0.2 ng/follicle).
Effect of GnRHa (1 μg/ml), LH (1 μg/ml) and GnRHa + LH on progesterone levels in the tissue (left panel) or incubation media (right panel) after 4 h incubation. The values shown are the mean ± SE of 4 observations per group and are from one typical experiment. A significant (P < 0.01) stimulation of progesterone levels in tissue and media were obtained in all hormone-treated groups. The effects of GnRHa + LH were not significantly higher than the effects of LH alone.

Experiments it was found that the effect of GnRHa (10 ng/ml) was abolished by the GnRH antagonist (10 μg/ml) (control 0.20 ± 0.06, GnRHa 3.23 ± 0.27, GnRHa + antagonist 0.20 ± 0.10 ng progestrone per follicle). The time-course of progesterone accumulation in response to GnRHa is demonstrated in Fig. 2. A significant stimulation over control was found at 4 and 6 h of incubation but not at 2 h of incubation.

The levels of progesterone were found to increase in the tissue as well as in the incubation medium (Fig. 3). The tissue levels were similar in the presence of LH or GnRHa but the medium levels were approximately 10-fold higher in the presence of LH as compared to GnRHa. There appeared to be no additivity between LH and GnRHa on progesterone accumulation (Fig. 3 and Table 2).

Table 1.
Effect of GnRHa and LH upon follicular cAMP.

| Treatment | Tissue levels | | Medium levels | |
|-----------|---------------|------------------|------------------|
|           | 30 min | 60 min | 120 min | 30 min | 60 min | 120 min |
| Control   | 66 ± 4   | 57 ± 9   | 50 ± 5   | < 40    | 54 ± 21 | < 40    |
| GnRHa     | 66 ± 15  | 68 ± 5   | 65 ± 5   | < 40    | < 40    | < 40    |
| LH        | 1343 ± 152 | 2872 ± 396₁  | 1488 ± 179₁  | < 40    | 684 ± 271₁  | 1105 ± 473₁  |

Follicles were incubated for 30-120 min with or without GnRHa (1 μg/ml) or LH (1 μg/ml). The levels of cAMP in the follicular tissue and incubation medium was determined separately and are expressed as pmol/mg protein. The values shown are the means ± SE of 4 observations per group and are from one typical experiment.

₁ P < 0.01 vs control.
Table 2.
Effect of GnRHa and LH alone and in combination upon follicular cAMP level and progesterone secretion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP (pmol/mg protein)</th>
<th>Progesterone (ng/follicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Control</td>
<td>106 ± 8</td>
<td>137 ± 20</td>
</tr>
<tr>
<td>GnRHa</td>
<td>124 ± 14</td>
<td>267 ± 23&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>LH</td>
<td>1351 ± 74&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1194 ± 174&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>GnRHa + LH</td>
<td>1725 ± 142&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1219 ± 138&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Follicles were incubated for 2 or 4 h with or without GnRHa (1 μg/ml) or LH (1 μg/ml). cAMP levels in the tissue and progesterone levels in the medium are given. The data are pooled from two different experiments and are the means ± SE of 4–8 observations per group.

<sup>1</sup> P < 0.01 vs appropriate control with analysis of variance after log transformation.

**cAMP formation**

There were no detectable changes in cAMP levels in follicular tissue or medium in the presence of GnRHa during 30–120 min of incubation (Table 1). By comparison, LH produced a marked stimulation with peak levels in the tissue after 60 min of incubation. At 4 h of incubation, however, GnRHa caused a small but significant stimulation of cAMP levels in the follicles (Table 2). This pattern of response to GnRHa was not changed by MIX (0.1 mm), which was tested for incubation times varying between 15–240 min (data not shown).

**Prostaglandin accumulation**

GnRHa and LH both significantly stimulated PGE accumulation at 4 h of incubation (Table 3). Indomethacin abolished the rise in PGE induced by both hormones but had no effect on the response in terms of progesterone or oocyte meiosis. Indomethacin also had no effect on the slight elevation of cAMP at 4 h of incubation.

Table 3.
Effect of GnRHa and LH on follicular prostaglandin and progesterone release and on oocyte maturation, with or without indomethacin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prostaglandin E</th>
<th>Progesterone</th>
<th>cAMP&lt;sup&gt;3&lt;/sup&gt; (pmol/mg protein)</th>
<th>Oocyte maturation (% GVB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59 ± 8 (9)</td>
<td>0.46 ± 0.06 (5)</td>
<td>110 ± 11 (3)</td>
<td>0 (50)</td>
</tr>
<tr>
<td>I</td>
<td>53 ± 13 (10)</td>
<td>0.77 ± 0.10 (5)</td>
<td>113 ± 18 (3)</td>
<td>0 (49)</td>
</tr>
<tr>
<td>GnRHa</td>
<td>247 ± 50 (10)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.85 ± 0.20 (5)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>260 ± 20 (3)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>96 (50)</td>
</tr>
<tr>
<td>GnRHa + I</td>
<td>62 ± 22 (10)</td>
<td>1.82 ± 0.26 (5)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>365 ± 47 (3)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>86 (50)</td>
</tr>
<tr>
<td>LH</td>
<td>290 ± 31 (10)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>18.6 ± 3.4 (4)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1844 ± 177 (3)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>98 (47)</td>
</tr>
<tr>
<td>LH + I</td>
<td>52 ± 13 (10)</td>
<td>23.8 ± 2.0 (5)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1550 ± 98 (3)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>94 (50)</td>
</tr>
</tbody>
</table>

Follicles were incubated for 4 h ± indomethacin (I) (3 × 10<sup>−4</sup>M). GnRHa and LH were used in concentration of 1 μg/ml. The accumulation of prostaglandin E and progesterone in the medium is given as the mean ± SE and (n) indicates number of observations. Oocyte maturation was recorded by Nomarski interference contrast microscopy and the percentage showing germinal vesicle breakdown (GVB) of the total number examined (within parenthesis) is given. 3 cAMP in the tissues was determined in a separate experiment.

<sup>1</sup> P < 0.05. 2 P < 0.01 vs control with analysis of variance.
Discussion

Our results show that GnRH and two potent analogs directly stimulate follicular progesterone and prostaglandin E accumulation in vitro, principally confirming the data obtained in isolated rat granulosa cells during short-term incubation (Clark et al. 1980; Clark 1982). When compared to LH, however, the actions of GnRH differed in some important ways: 1) the maximal effect on progesterone was approximately 10-fold lower for GnRH than for LH; 2) the time-course of progesterone stimulation was different, with GnRH being effective only a 4 and 6 h, but not at 2 h of incubation, whereas LH causes a marked increase within 2 h under these conditions (Hamberger et al. 1978). 3) no changes in follicular cAMP levels could be detected between 15 and 120 min of incubation and only at 240 min of incubation could a slight, yet significant, elevation of cAMP be detected. This is in contrast to LH which, as expected, caused a marked and prolonged increase in cAMP that was observed already at 15 min of incubation, and which is consistent with previous reports (Nilsson et al. 1974). It cannot be excluded that the increase in progesterone secretion caused by GnRH is mediated by cAMP. On the other hand it appears unlikely that GnRH-induced oocyte meiosis, observed already at 2 h of incubation, is mediated by cAMP (Hillensjö & LeMaire 1980).

The stimulatory effect of GnRH on follicular prostaglandin accumulation is quite similar to the effect of LH and both effects are abolished by indomethacin. These findings are consistent with the observations that GnRH induced ovulation, like LH induced ovulation in hypophysectomized immature rats is inhibited by indomethacin (Ekholm et al. 1982). From the data in Table 3 it is clear that the increase in prostaglandin E, induced by either LH or GnRH, does not play a role in progesterone accumulation, cAMP accumulation or oocyte maturation in these isolated follicles.

The acute stimulatory effects by GnRH upon follicular progesterone and prostaglandin accumulation differ from the more commonly known inhibitory effects on steroidogenesis reported by others. These discrepancies may, however, be more apparent than real due to differences in experimental models and approaches. As mentioned above most inhibitory effects on steroidogenesis are observed after prolonged exposure to GnRH and in connection with gonadotrophin stimulation, whereas we have analyzed the acute effect of GnRH on basal events rather than gonadotrophin-stimulated events. Since the stimulatory effect of GnRH on progesterone accumulation in whole follicles, as observed here, or in isolated granulosa cells, as reported previously (Clark & Marsh 1980; Clark 1982) is of small magnitude it may easily be overlooked and regarded as non-significant as compared to the pronounced effect of LH. Nevertheless, the fact that GnRH may have initial stimulatory effects on the ovarian follicle may be a significant finding in explaining the mechanism of GnRH action on the ovary. Other investigators have also demonstrated stimulatory effects of GnRH on ovarian function, including the stimulation of progesterone accumulation (Knecht et al. 1981; Jones & Hsueh 1981), morphologic luteinization (Hsueh et al. 1980), cyclic nucleotide production (Knecht et al. 1981) and oocyte maturation (Hillensjö & LeMaire 1980; Hsueh et al. 1980).

In the pituitary gland convincing evidence exists to suggest that Ca\(^+\) may be the second messenger in GnRH stimulation of gonadotrophin secretion (reviewed by Conn et al. 1981). Whether a similar mechanism of action for GnRH exists in ovarian tissue is presently unknown. The possibility that GnRH may trigger oocyte meiosis through changes in cellular free Ca\(^+\) is particularly intriguing since it has been shown that the ionophore A23187 may trigger meiosis in follicle-enclosed rat oocytes (Tsafrriri & Bar Ami 1978), probably through activating Ca-fluxes. In the amphibian oocyte recent data strongly indicate Ca\(^+\) as a second messenger in progesterone-induced meiosis (Moreau et al. 1980; Wasserman & Smith 1981). There is also some evidence for a role of Ca\(^+\) the regulation of steroidogenesis in adrenal tumor cells (Hall et al. 1981). Further work is needed to explain the cellular mechanisms involved in the acute stimulatory effects of GnRH on the preovulatory follicle. Further work is also needed to establish whether the direct effects of exogenous GnRH represent only pharmacological effects or represent indeed physiological events exerted through locally formed regulatory peptides.

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References


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