Adenylate cyclase activity in rat corpora lutea evidence for a rapid development of the regulatory Ns-protein

Sten Rosberg, Ensio Norjavaara, Monica Sender Baum and Iqbal Khan

Department of Physiology, University of Göteborg, Göteborg, Sweden

Abstract. Adenylate cyclase activity was studied in membranes from isolated corpora lutea of defined ages obtained from pregnant mare’s serum gonadotropin treated rats and the effects of luteinizing hormone (LH), isoproterenol, guanylylimidodiphosphate (Gpp(NH)p), fluoride and forskolin were compared. The effect of LH on adenylate cyclase activity increased with the luteal age up to nine days of age, while the effect of isoproterenol increased dramatically during the first days, reaching a maximum at 2–3 days of age and then declined. Forskolin potentiated the effects of both LH and isoproterenol without affecting the patterns of age-dependency. The effect of forskolin itself was fairly constant during the luteal phase, indicating a relatively constant amount of the catalytic unit in the corpus luteum. The effects of fluoride and Gpp(NH)p on the other hand increased markedly during the first days and then remained constant for the rest of the period studied. These results suggest that the regulatory Ns-protein develops during the first days of luteal life. It is speculated that the close correlation between the development of β-adrenergic response and the development of Ns-protein are causally related.

The ovary is a complex organ consisting of several compartmental structures and undergoing cyclic changes. One compartment, the corpus luteum, is formed from the pre-ovulatory follicles after the gonadotropin surge and, depending on the species, produces progesterone for a longer or shorter period of time until luteolysis (corpus luteum regression) occurs (Rothchild 1981). The regulation of the progesterone production that is necessary for normal gestation is dependent on luteotropic factors with a wide interspecies variation (Rothchild 1981). During the life-span of the corpus luteum, several important changes are seen in the response to luteotropic factors. In the pseudo-pregnant rat there is a gradual increase in LH receptor number (Lee et al. 1975; Richards & Midgley 1976; Rajaniemi et al. 1977) and in responsiveness to LH (Birnbaumer et al. 1976; Ahrén et al. 1981) with luteal age, while the β-adrenergic receptor content as well as the responsiveness to catecholamines increase steeply for the first few days whereafter it declines (Norjavaara et al. 1984; Selstam et al. 1984). Furthermore, there is a marked shift in the sensitivity to the luteolytic action of prostaglandin F2α (PGF2α), i.e. PGF2α can abrogate the actions of both LH and catecholamines more readily in older corpora lutea than in younger ones (Khan et al. 1979; Ahrén et al. 1983).

These dramatic changes during the life-span of the corpora lutea have initiated us to study more closely the adenylate cyclase system in corpora lutea at various stages of development. Apart from hormones, we have used agents which are able to influence the adenylate cyclase at various points distal to the hormonal receptors.

Materials and Methods

Hormones and chemicals
LH (NIH-I.H-S19) was kindly supplied by the National Institute of Health, Bethesda, MD. L-Isoproterenol (Iso)
was obtained from Sigma, St. Louis, MO. α-[32P]ATP (30 Ci/mmol) and [3H]cAMP (26 Ci/mmol) were purchased from Amersham International, Buckinghamshire, UK. The guanylyl nucleotides, GTP and guanylylimidodiphosphate (Gpp(NH)p), were purchased from Boehringer Mannheim, West Germany and forskolin from Calbiochem-Behring Corp., La Jolla, CA. All other chemicals were of analytical grade and purchased either from Merck, Darmstadt, FRG or Sigma, St. Louis, MO.

**Animals**

Immature Sprague-Dawley rats, purchased from ALAB, Stockholm, Sweden, were kept under controlled conditions (25°C and 60% humidity with a light period between 05.00 h and 07.00 h) and fed a standard pellet diet with free access to tap water.

**Corpus luteum model**

A well characterized rat ovulatory model was used where follicular maturation and ovaulations are induced shortly before normal puberty (Herlitz et al. 1976). In this model a single dose of 8 IU of pregnant mare’s serum gonadotropin (PMSG, Sigma) is injected sc in the neck skin of the rat in the morning (08.00 h-10.00 h) when the rats are 26 days old. This treatment is known to induce follicular maturation, and in the afternoon of day 28 a preovulatory endogenous surge of gonadotropins occurs, resulting in ovulation early in the morning (around 02.00 h) of day 29. On day 29, ovaulations are easily recognized in a stereo-microscope by the presence of newly formed corpora lutea. The length of the luteal phase is approximately 10–12 days and about 14 corpora lutea are formed per rat (Norjavaara et al. 1984).

After decapitation of the rats, the ovaries were rapidly excised and placed in ice-cold buffer. The corpora lutea were dissected free with the help of syringe needles under a stereo-microscope. All corpora lutea from each rat were frozen on dry ice and kept at −70°C until analysed for adenylate cyclase activity. Only corpora lutea from rats not showing luteolytic signs, as judged by the plasma progesterone and 20α-dihydroprogesterone values, were used for the adenylate cyclase analysis.

**Adenylate cyclase assay**

Membranes for the adenylate cyclase assay were prepared by homogenizing the corpora lutea from each rat in ice-cold Tris buffer (10 mM Tris and 1 mM EDTA, pH 7.5) with 27% sucrose (Birnbaumer et al. 1976). The homogenate was centrifuged for 5 min at 160 × g to remove debris, filtered through two layers of cheese cloth and centrifuged again for 50 min at 10 000 × g. The supernatant was discarded and the crude membrane pellet resuspended in the Tris-sucrose buffer. Aliquots of 100 μl of this suspension (corresponding to 25–100 μg protein) were used in the adenylate cyclase assay. The final concentrations in the adenylate cyclase assay were as follows: 3 mM ATP (with approximately 106 cpm [32P]ATP), 10 mM MgCl2, 20 mM creatine phosphate, 1 mg/ml creatine phosphokinase and 1 mM cAMP in 50 mM Tris buffer at pH 7.5. The reaction was started by the addition of 100 μl membrane suspension to 100 μl of the incubation medium. The assay was performed at 37°C for 15 min and stopped by the addition of 100 μl of a stopping solution (5 mM CAMP, 20 mM ATP and 1% Na-dodecysulfate). The reaction was linear at least up to 60 min, except in the presence of Gpp(NH)p where a slight lag phase was seen. The [32P]cAMP formed was isolated by Dowex and alumina column chromatography according to Salomon et al. (1974). The recovery of each sample was monitored by addition of [3H]cAMP. The eluates from the alumina columns were collected directly into scintillation vials, mixed with scintillation fluid and counted in a scintillation spectrometer. Protein content of the membrane suspension was determined according to the method of Lowry et al. (1951) after precipitating with ice-cold 10% trichloroacetic acid.

**Statistical analysis**

Comparisons were made with one or two-way analysis of variance, or, when appropriate, with a randomized block design, followed by Student-Newman-Keul’s multiple range test (Wooll 1968).

**Results**

In the first part of the study, the effects of LH and isoproterenol in combination with various concentrations of forskolin were compared. As seen in Fig. 1 there was a marked difference in the patterns of stimulation of LH and isoproterenol. Isoproterenol gave maximal stimulation of adenylate cyclase activity in 3-day-old corpora lutea, while with LH a gradual increase in adenylate cyclase activity was seen with luteal age up to nine days of age. Forskolin, in the two highest concentrations, potentiated the effects of both LH and isoproterenol with about 100% without affecting the patterns of age dependency (Table 1). Forskolin, by itself, markedly stimulated the luteal adenylate cyclase activity. However, for this drug no apparent shift in sensitivity with the luteal age was seen, except for a downward trend at the highest concentration of forskolin (Fig. 1).

In the next part of the study, the influences of fluoride and Gpp(NH)p on luteal adenylate cy-
clase activity were also investigated. As seen in Fig. 2, there was a marked increase in the activation by both fluoride and Gpp(NH)p during the first 2–3 days (i.e. 7, 19 and 31 h after ovulation, corresponding to the 3 first time points, respectively). This increase coincides closely with the increase

**Table 1.**
Effect of different forskolin concentrations on the responsiveness to LH and isoproterenol. The responsiveness of the hormones are given as Δ-responses (total activity – respective control) in the presence of the indicated forskolin concentrations.

<table>
<thead>
<tr>
<th>Corpus luteum age (days)</th>
<th>Δ-response of adenylate cyclase activity (pmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>LH</td>
<td>2 ± 4</td>
</tr>
<tr>
<td>LH + forskolin 1 µM</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>LH + forskolin 10 µM</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>LH + forskolin 100 µM</td>
<td>29 ± 20</td>
</tr>
<tr>
<td>Iso</td>
<td>107 ± 10</td>
</tr>
<tr>
<td>Iso + forskolin 1 µM</td>
<td>178 ± 10</td>
</tr>
<tr>
<td>Iso + forskolin 10 µM</td>
<td>213 ± 32</td>
</tr>
<tr>
<td>Iso + forskolin 100 µM</td>
<td>268 ± 15</td>
</tr>
</tbody>
</table>

The data from Fig. 1 are expressed as Δ-responses to LH and isoproterenol (Iso), respectively. The Δ-responses are calculated as the response to the hormone in combination with the respective forskolin concentrations (or control medium) minus the response to forskolin alone.
seen with isoproterenol during the first luteal day. However, the response to isoproterenol decreased after three days of age, while the responses to fluoride and Gpp(NH)p were approximately constant throughout the rest of the luteal period. The activation by forskolin was constant during the first days followed by a slight decrease later on. The activation by forskolin and GTP together was always higher than that of forskolin alone, and the combination of forskolin and Gpp(NH)p gave approximately an additive activation throughout the luteal life (Fig. 2).

Discussion

The results reported in the present demonstrate that there are intricate changes in the receptor-adenylate cyclase system of the rat corpus luteum with luteal age. The hormone responsive adenylate cyclase system consists of three principal components: the catalytic unit, the hormonal receptors and the regulatory nucleotide binding proteins (N-proteins) (Birnbaumer et al. 1985). Each of these components may be subject to changes with the age of the corpus luteum, thus leading to a complex response pattern.

In an attempt to elucidate the relative importance of the different adenylate cyclase components, we have used agents which are believed to mainly interact at specific parts of the adenylate cyclase system. The diterpene forskolin was originally believed to interact mainly with the catalytic unit (Seamon & Daly 1981), but later evidence indicate that forskolin also, to some degree, can interact with the N-proteins (Birnbaumer et al. 1985). In the present study, the activation by forskolin of the adenylate cyclase was approximately constant for the different luteal ages. This finding may be an indication that the number of catalytic units does not change during the luteal

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Fig. 2.
Age dependency of adenylate cyclase activity. Adenylate cyclase was assayed in membranes of corpora lutea of the indicated ages with 3 mM ATP and 10 mM MgCl₂. In the left panel the adenylate cyclase activity was assayed in the presence of GTP alone (50 µM) or with GTP and LH (1 µg/ml), isoproterenol (5 µg/ml), NaF (10 mM) and forskolin (100 µM), respectively. In the right panel the enzyme activity was assayed in the presence of Gpp(NH)p (100 µM), forskolin (100 µM) and the combination of these two agents. The values shown are means ± SEM of 3–6 observations (rats).
period. On the other hand it is known that the receptors for both LH and catecholamines vary with the luteal age. Thus, the number of LH receptors increases with the luteal age (Lee et al. 1975; Richards & Midgley 1976; Rajaniemi et al. 1977), while the number of catecholamine receptors increases steeply for the first few days and then declines (Norjavaara et al. 1984). These patterns of hormone receptor development coincide closely with the response patterns of the adenylate cyclase to the respective hormones reported here.

The third component of the adenylate cyclase system, the N-proteins, are found in two independent forms, one stimulatory (N\(_s\)) and one inhibitory (N\(_i\)), and both of these can be activated by Gpp(N)p and fluoride (Spiegel et al. 1981; Birnbaumer et al. 1985). We show here that the responses to both of these two substances increase markedly during the first luteal days and then remain more or less constant for the rest of the period studied. One interpretation of this finding may be either that the inhibitory influence of the N\(_s\)-protein is diminished or that the amount of the stimulatory N\(_s\)-protein in the corpus luteum is increased markedly during the first day. The latter view is supported by a recent finding of McIlroy & Bergert (1984) who measured in a more direct way by means of \(^{32}\)P]ADP-ribosylation the amount of N\(_s\)-protein in ovarian membranes from PMSG/hCG-primed rats. These authors reported a marked increase in the amount of N\(_s\)-protein during the first days after hCG treatment. In their study, McIlroy & Bergert (1984) also showed a close correlation between the response to isoproterenol, the amount of \(\beta\)-receptors and the amount of N\(_s\)-protein. In corroboration with their study, we also find a close correlation between the development of the response to isoproterenol and the development of responses to Gpp(NH)p and fluoride. To our knowledge, the presence of an inhibitory N-protein in luteal tissue has only been reported for the rabbit (Abramowitz & Campbell 1984). These authors demonstrated that GTP and Gpp(NH)p dose-dependently inhibited the activation of rabbit adenylate cyclase forskolin. However, concentrations of GTP and Gpp(NH)p that were maximally inhibitory in the rabbit corpus luteum (Abramowitz & Campbell 1984) did not inhibit the forskolin activated adenylate cyclase of the rat luteal membranes. Rather, there was a synergistic effect of these compounds at all luteal ages studied (Fig. 2). Furthermore, in preliminary experiments we have found no evidence for a dose-dependent inhibition by GTP or GTP-analogues of forskolin-activated rat luteal adenylate cyclase (Hedin et al., unpublished). Thus, there appears to be a species difference between the rat and the rabbit corpus luteum in this respect, and the existence of a N\(_s\)-protein in the rat corpus luteum remains to be explored.

The fact that catecholamines can stimulate adenylate cyclase and steoridogenesis in corpora lutea has become evident during the recent ten years (Selstam et al. 1984; Norjavaara 1984), but the physiological relevance of this stimulation is still an enigma. The close correlation between the amount of N\(_s\)-protein on the one hand, and the \(\beta\)-adrenergic receptors and \(\beta\)-adrenergic effects on adenylate cyclase on the other during the first luteal days, makes it tempting to suggest that these two components, the N\(_s\)-protein and the \(\beta\)-receptor, are transferred to the cell membrane in a coordinated way. The increase in \(\beta\)-adrenergic receptors could thus reflect a way for the luteal cells to rapidly increase the amount of N\(_s\)-protein in the cell membrane. When the amount of \(\beta\)-adrenergic receptors declines later on, the N\(_s\)-protein would be left intact, available to interact with the increasing amount of LH receptors.

The development of the N\(_s\)-protein in the young corpora lutea is of interest from another point of view, namely the resistance of the newly formed corpora lutea to the luteolytic effects of PGF\(_{2\alpha}\) (Rothchild 1981). We have earlier shown that incubated 1-day-old corpora lutea are totally resistant to the inhibitory effects of PGF\(_{2\alpha}\) on LH stimulation (Khan et al. 1979) as well as on epinephrine stimulation (Ahrén et al. 1983), while in 3-day-old corpora lutea or older, PGF\(_{2\alpha}\) could inhibit the effects of both LH and epinephrine. This might indicate that the N\(_s\)-protein is one component of importance for expressing the inhibitory/luteolytic actions of PGF\(_{2\alpha}\).

In conclusion, the results of the present support the view that there is a rapid development of the stimulatory N-protein during the first few days of luteal life, while the amount of the catalytic units seems to be more or less constant throughout the luteal period. Future experiments with ADP-ribosylation will hopefully give the quantity and the relative proportion of the stimulatory and inhibitory N-proteins in luteal tissue of various ages.
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References


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