Cyclic AMP phosphodiesterases of the rat ovary. 
Oestrous cycle dependent activity change of high affinity form

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Abstract. In the rat ovary, from prenatal to adult stages, two electrophoretically distinct forms of cyclic AMP phosphodiesterase (E.C.: 3.1.4.17) occur. These forms were analysed for their affinity towards cAMP in both crude and partially purified preparations. Whereas the slow electrophoretic form, which has a low affinity towards cAMP, shows a linear Lineweaver-Burk plot, the fast electrophoretic form exhibits anomalous kinetic behaviour in that cAMP affinity appears to increase with decreasing cAMP-concentration. This form shows dramatic alteration of maximum velocity during the oestrous cycle, attaining maximum activity at metoestrous. This rise of activity is sensitive to inhibition of protein synthesis.

In the rat, as well as in other mammals, a dramatic surge of luteinizing hormone (LH) and follicle stimulating hormone (FSH) occurs during the pre-oestrous stage of the ovarian cycle (Döhler & Wuttke 1975). Many actions of these hormones on the ovary are apparently mediated by cyclic AMP (cAMP) (for references see Marsh 1975). The role of this nucleotide in the regulation of steroidogenesis, luteinization, ovum maturation and ovulation has recently been reviewed (Hunzicker-Dunn et al. 1979). As in other systems showing hormone-dependent elevation of cAMP, also in the ovarian follicle, the increase of cAMP is only transient (Hunzicker-Dunn et al. 1979). The decline of cAMP concentration following its stimulation may, in principle, either be due to a fall in adenyl cyclase activity or to a specific stimulation of cAMP phosphodiesterase (PDE, E.C.: 3.1.4.17), known to represent the only intracellular specific cAMP catalyzing activity, or to alterations of both of these enzyme activities. In this report we describe kinetic and electrophoretic properties of rat ovarian PDE's, present a simple method of separating electrophoretically and kinetically distinct forms, and show that a specific enzymatic form with high cAMP affinity exhibits oestrous cycle stage dependent alterations of activity.

Materials and Methods

Animals
Rats of the strain SIV-50 were used. The animals were housed in air-conditioned and light-controlled rooms (light period from 6.00 a.m. to 9.00 p.m.). The animals received water and standard rat chow (Altromin 1314, D-4937 Lage, FRG) ad libitum. Dates of pregnancy were determined by the presence of a vaginal plug.

Control of cycle stage
Cyclus stage was determined by vaginal smears, which were examined by phase microscopy as well as after Papanikolaou staining. Accordingly, the animals were classified into the five stages of the oestrous cycle, namely: pro-oestrus, oestrus, metaoestrus, dioestrus I and II. The pro-oestrus stage was also marked by radioimmunoassay of serum LH, kindly carried out by Dr. W. Wuttke, Göttingen.

Preparation of ovarian homogenates
Animals were killed by decapitation; the trunk blood was collected, and serum was withdrawn by centrifugation, serving for the LH-radioimmunoassay. The ovaries were removed, trimmed of fat and adhering connective tissue, and then processed according to the method of Thomp-

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son & Appleman (1971). Homogenization was performed at 0°C in 0.32 M sucrose – 0.9% NaCl at a proportion 1:10 (tissue weight: volume medium) using 30 strokes in a motor-driven glass/glass Potter-Elvehjem homogenizer. This crude homogenate was sonicated with a Branson sonific B 12 cell disruptor at setting 4 (100 W) for 15 s, adjusted to pH 6.0 with 0.1 M acetic acid and was finally centrifuged at 20 000 × g for 20 min. The supernatant was used for all kinetic experiments with crude homogenates. For electrophoretic analysis, ovaries were homogenized 1:1 (w/v) in 50 mM Tris – 1 mM MgSO4, pH 7.4, sonicated as above, but centrifuged at 100 000 × g for 1 h at 0°C in a Beckman L5-65 ultracentrifuge. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

**Electrophoresis of PDE**

100 000 × g supernatants were applied to gels made of 12.5% starch (Connaught Lab., Canada) in 0.01 M Tris – 0.01 M malic acid – 0.001 M MgCl₂ (pH 7.7 adjusted with NaOH); bridge 0.1 M Tris – 0.1 M malic acid – 0.01 M MgCl₂ (pH 7.7). Gels were run horizontally at 5 V/cm for 10–16 h. Staining of gel slices was done according to the method of Monn & Christiansen (1971). Chemicals and stain reagents were from Boehringer, Mannheim, FRG, and Merck, Darmstadt, FRG.

**Isolation of PDE-Isozymes**

For preparative purposes, PDE was electrophoresed as above, areas of the gel block corresponding to stained zones were excised and homogenized in 10.9% sucrose – 0.9% NaCl – 1% cytochrome C (Boehringer) using a motor-driven glass/glass homogenizer. The maintenance of a high protein concentration by the addition of cytochrome C prevented recovery losses. Cytochrome C does not interfere with the PDE assay; furthermore, due to its small molecular weight, it does not become strongly enriched during the following concentration procedure (see below). After homogenization of the starch, the gel mass was centrifuged off (1 h, 45 000 × g), and the supernatant was treated with α-amylase for 60 min at ambient temperature (40 μg α-amylase lyophilisate (Boehringer) per 20 ml gel supernatant). Thereafter the supernatant was concentrated to the desired PDE activity by ultrafiltration (Amicon PM 30 membranes).

**PDE Assay**

The PDE assay was similar to that of Rutten et al. (1973) with minor modifications (Schmidtke et al. 1976). A final volume of 140 μl contained 6.6 mM Tris-HCl, pH 8.5, 1 mM MgCl₂, 0.18 μM [3H]cAMP (Amersham, 26 Ci/mmol), 0.25–360 μM non-labelled cAMP (Boehringer), and 25 μl appropriately diluted probe. Incubation at 37°C was usually terminated after 20 min, by immersing the reaction vials into boiling water for 2 min. After addition of 8 μl 5′-nucleotidase (Sigma), the mixture was incubated at 37°C for 30 min, centrifuged at 10 000 × g for 2 min, and finally passed over Dowex 1X8, 200–400 mesh (Serva, Heidelberg, FRG). Elution of cAMP breakdown products, which is complete under these conditions (Rutten et al. 1973), was done with 0.1 M NaHCO₃. Two ml of the eluate were mixed with a suitable scintillation cocktail and counted in a Tri-Carb 2660 liquid scintillation spectrometer.

**Evaluation of the data**

Enzyme kinetics in which two kinetically distinct molecular forms of PDE were acting simultaneously on cAMP, as judged from electrophoresis and curved Lineweaver-Burk plots, were evaluated using the computer-aided method of Osmundsen (1975). Kinetics of isolated PDE forms, which yielded a linear Lineweaver-Burk plot, were calculated by a direct computer-fitting of the data to the Michaelis-Menten equation, using the BMDX-Non Linear Least Squares computer programme.

**Fig. 1.**

Starch-gel electrophoresis of adult rat ovary (left lane) phosphodiesterase. Two distinct electrophoretic forms are seen in the ovary preparation. This pattern does not change substantially during development (late prenatal-adult stages). Adult lung phosphodiesterase exhibiting a three-banded electrophoretic pattern is shown for comparison in the right lane.
Results

Electrophoretic and kinetic characterisation of rat ovary PDE.

Starch-gel electrophoresis of adult rat ovarian homogenate yields two distinct bands (Fig. 1). There is no developmental stage dependent variation of this isoenzyme pattern; it remains unchanged throughout the entire developmental period tested (19th prenatal to adult stages; data not shown).

Kinetic experiments were performed in the range from 0.36 – 0.0004 mM cAMP using crude ovarian homogenate of prenatal (20th day of pregnancy), 23-day-old, 45-day-old, and adult cyclic animals. At all developmental stages two distinct kinetic components were apparent. Table 1 lists the apparent Michaelis constants. It is seen that during the entire development an enzymatic form with very high affinity towards cAMP (2.2 – 3.8 μM) co-exists with a form of an affinity two orders of magnitude lower (150 – 290 μM). In Fig. 2, a typical Lineweaver-Burk plot of the relationship between cAMP concentration and reaction velocity in adult ovarian homogenate (dioestrous II-stage) yielding a concave downward curve is shown.

In order to test whether the two kinetic components correspond to the two isoenzymic forms distinguishable electrophoretically, the latter were isolated from each other by the procedure described above, using adult ovarian tissue homogenate. Fig. 3 demonstrates that the isolated 'slow' enzyme yields a linear Lineweaver-Burk plot, whereas the isolated 'fast' form exhibits anomalous kinetic behaviour in that the affinity towards cAMP appears
Table 1.
Apparent Michaelis constants of rat ovarian cAMP phosphodiesterases at various developmental stages.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Low K_m [μM]</th>
<th>High K_m [μM]</th>
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<tbody>
<tr>
<td>Prenatal (20th day of pregnancy)</td>
<td>2.15±0.63</td>
<td>150±2.22</td>
</tr>
<tr>
<td>23rd day</td>
<td>2.88±0.63</td>
<td>170±2.22</td>
</tr>
<tr>
<td>45th day</td>
<td>3.67±0.63</td>
<td>250±2.22</td>
</tr>
<tr>
<td>Adult</td>
<td>3.82±0.42</td>
<td>290±3.02</td>
</tr>
<tr>
<td>Adult (isolated from gels)</td>
<td>36.8±6.0</td>
<td>130±9.0</td>
</tr>
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</table>

1 Mean and SEM of the 26 experiments of Fig. 4 are given.
2 ‘Fast’ electromorph.
3 ‘Slow’ electromorph.

In order to test whether the activities of ovarian cAMP PDE’s vary during the oestrous cycle, V_max and K_m of high and low K_m PDE were estimated in 26 rats at different cyclus stages. No significant change of the K_m was observed for either form. The maximum velocities are presented in Fig. 4.

Lineweaver-Burk plot of the effect of substrate concentration on the velocity of cAMP phosphodiesterase in isolated ‘slow’ (○) and ‘fast’ (■) isoenzymes of adult rat ovary. Isolation was performed by preparative starch-gel electrophoresis. The kinetic parameters are given in Table 1. The reaction velocity is given on an arbitrary scale, because the enzyme preparations contained excess exogenous protein (see ‘Material and Methods’).
cAMP phosphodiesterase activity in crude rat ovary homogenates at various stages of the ovarian cycle (P = pro-oestrous; E = oestrous; M = metoestrous, D I, II = dioestrous I, II). Mean ± SEM of maximum velocities of high Km (filled bars) and low Km (open bars) forms are given. Figures in the bottom of each bar indicate the number of experiments. Each experiment was done with the ovaries of a single animal. The increase of low Km phosphodiesterase activity at metoestrous is highly significant (P < 0.001) as compared to the pro-oestrous value.

With regard to the high Km ovarian PDE no oestrous cycle stage dependent alterations of activity were observed. The slight differences in maximum velocity seen were not statistically significant as revealed by variance analysis. However, there is a dramatic increase of the low Km ovarian PDE activity during the metoestrous stage of the cycle. This increase is highly significant compared with the pro-oestrous value (t-test; P < 0.001). All other stages are homogeneous with respect to low Km PDE activity.

The question whether the increase of low Km PDE activity is dependent on de novo enzyme synthesis was tested in the following way. Rats at the pro-oestrous stage were injected with actinomycin D and cycloheximide (Serva, Heidelberg, FRG), both of which are effective inhibitors of protein synthesis; actinomycin D primarily blocks transcription of mRNA. Control animals received physiological saline solution. The animals were killed at the metoestrous stage, and PDE activity was measured at both high (0.36 mM) and low (0.4 μM) cAMP concentration. As shown in Table 2, at a dose of 1.5 μg/g body weight of actinomycin D, PDE activity measured at both cAMP concentrations is effectively reduced. It appears that the effect of the toxin is more pronounced with the high Km activity, which dominates the reaction at high substrate concentration. A similar effect is seen when cycloheximide (2–4 μg/g body weight) is given (Table 2).

### Table 2.

<table>
<thead>
<tr>
<th></th>
<th>cAMP-PDE activity (nmoles cAMP hydrolysed/mg protein/20 min) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (physiological saline)</td>
<td>0.36 mM cAMP 0.4 μM cAMP</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>77.8 ± 8.1 0.52 ± 0.06</td>
</tr>
<tr>
<td>(1.5 μg/g body weight)</td>
<td>8.5 ± 2.8 0.15 ± 0.02</td>
</tr>
<tr>
<td>Cycloheximide (2 μg/g body weight)</td>
<td>39.6 ± 8.6 0.45 ± 0.12</td>
</tr>
<tr>
<td>Cycloheximide (4 μg/g body weight)</td>
<td>26.3 ± 4.4 0.31 ± 0.07</td>
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Animals were injected with the toxin indicated at the pro-oestrous stage and killed at metoestrous.
Table 3.
cAMP phosphodiesterase activity in mixtures of pro-oestrous and metoestrous rat ovary homogenates.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pro-oestrus</th>
<th>Metoestrous</th>
<th>Mixture</th>
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<tbody>
<tr>
<td>No. 1</td>
<td>0.67</td>
<td>0.90</td>
<td>0.78</td>
</tr>
<tr>
<td>No. 2</td>
<td>0.58</td>
<td>0.96</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Values given are means of duplicate determinations of phosphodiesterase activity at 0.4 μM cAMP expressed in nmoles cAMP hydrolysed/mg protein/30 min. All samples were pre-incubated either alone or in mixture at 37°C for 30 min (experiment 1) or 45 min (experiment 2).

Since it appeared possible that the rise of low Km PDE activity is promoted by an inducible PDE activator (see 'Discussion'), experiments were performed with mixtures of metoestrous and pro-oestrous crude homogenates. It is shown in Table 3 that the PDE activity in pre-incubated mixtures measured at 0.4 μM cAMP is simply additive.

Discussion

The finding that two distinct electrophoretic forms of PDE can be separated from adult rat ovarian homogenate (Fig. 1) confirms an earlier report of Monn & Christiansen (1971). Contrary to the situation in rat testicular tissue exhibiting a pronounced developmental stage dependent variation of the PDE isoenzyme pattern (Epplen et al. 1980), the ovarian electrophoretic pattern is unaltered from prenatal to adult stages. Our kinetic experiments have shown that throughout these developmental stages enzymatic forms with high and low substrate affinity co-exist (Table 1, Fig. 2). Presence of low and high Km PDE's has also been described in corpora lutea of the bovine ovary (Stansfield et al. 1971; Michie et al. 1974; Goff & Major 1976). Our experiments designed to relate the electromorphs to the kinetically defined components have shown that the isolated slowly migrating electrophoretic form represents a kinetically homogeneous enzyme with low substrate affinity, whereas the fast electromorph exhibits anomalous kinetic behaviour (Fig. 3, Table 1). The decrease of affinity observed under increasing cAMP concentration suggests negative cooperativity, typical for purified cAMP PDE's also from other sources (Russel et al. 1972). This result, however, could also indicate the presence of a mixture of kinetically distinct PDE's within the fast electrophoretic form, not separated by the electrophoretic method used.

During the oestrous cycle the low Km PDE shows highly significant variation of activity (Fig. 4). At metoestrous this enzyme is about three times more active compared to the remaining cyclus stages. It appears that, following the surge of LH and FSH during pro-oestrous, at the metoestrous stage (about 24 h later), the PDE form with high substrate affinity is selectively induced. When this observation is related to ovarian cAMP levels, which are maximally about 2 μM (Nilsson et al. 1975) it becomes apparent that at this concentration cAMP catabolism is dominated by the low Km PDE activity, under the in vitro conditions described. The physiological implications of the apparent low Km PDE induction are dependent on its cellular site, as discussed below.

With the aim of clarifying the mechanism underlying the apparent induction of low Km PDE at metoestrous we were confronted with two queries: (1) is the rise in low Km PDE activity dependent on de novo enzyme synthesis? (2) is there any evidence as to an inducible PDE activity regulating factor? From the experiments using actinomycin D and cycloheximide as protein synthesis blocking agents (Table 2) we tentatively conclude that due to a rapid turnover of both forms of PDE, de novo synthesis of enzyme molecules is required for the rise of low Km PDE activity at the metoestrous stage. It is possible that the rise of low Km PDE activity is promoted by an inducible factor related to the PDE activator protein found in other tissues (Wells & Hardman 1977). A first simple approach to test this possibility, however, was unsuccessful: if such a factor would exist at higher concentration or activity during the metoestrous stage, a pre-incubated mixture of crude homogenates of metoestrous ovary with pro-oestrous ovary should not be simply additive with respect to low Km PDE activity, but rather it should mimic the activity typical for the metoestrous stage. As is seen in Table 3, two such experiments demonstrated that the PDE activity measured at low cAMP concentrations in mixture of pro-oestrous and metoestrous ovary homogenates was simply additive. Under the conditions employed, therefore, a PDE activator could not be demonstrated.
The alterations of low $K_m$ PDE activity in the ovary are obviously linked to the cyclic change of hypophysial hormone action producing alterations of cAMP concentrations. Involvement of PDE in the reduction of cAMP levels during the hormonally induced desensitized state of a cell has been suggested (Newcombe et al. 1975, and references cited therein). In vitro experiments with isolated Graafian follicles, however, have shown that the decline of cAMP levels is produced by desensitization of the adenylyl cyclase system to its stimulants, with no concomitant rise of PDE activity (Hunzicker-Dunn & Birnbaumer 1976; Lamprecht et al. 1977). Such experiments imply that the cAMP levels in Graafian follicles are beginning to decline before the PDE activity is measurably increased in whole ovaries (Lamprecht et al. 1977; Nilsson et al. 1975). A similar effect has been observed in PGE1 stimulated human synoviocytes suggesting a biphasic regulation of the hormonally induced events (Newcombe et al. 1975). It is possible that such a biphasic pattern does not occur under in vitro conditions in the follicle or that it has been overlooked.

On the other hand, it may be assumed that the cyclic fluctuation of a high affinity cAMP-PDE activity is not located in the mature follicle. It has been proposed, for example, that a follicular fluid inhibitor exists, which serves to prevent premature luteinization of granulosa cells in small follicles; there is evidence that such a factor acts by stimulation of a PDE activity (Channing 1979). Our findings may be related to these observations. Work is in progress aiming at the identification of the cellular site of the PDE activity changes while retaining the experimental conditions of the endogeneous cycle.

Acknowledgments

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References


Russel T R, Thompson W J, Schneider F W & Appleman...


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