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ADENOSINE 3',5'-CYCLIC PHOSPHATE PHOSPHODIESTERASE OF CORPUS LUTEUM

D. A. STANSFIELD, JANET R. HORNE AND G. H. WILKINSON
Department of Biochemistry, University of Dundee, Dundee (Great Britain)

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SUMMARY

1. Adenosine 3',5'-cyclic phosphate diesterase (Ado-3',5'-*P* phosphodiesterase) has been demonstrated in human, rat and bovine corpora lutea, and some properties of the bovine enzyme have been studied.

2. Bovine luteal Ado-3',5'-*P* phosphodiesterase is present in the 100 000 × *g* supernatant of tissue homogenate.

3. The mean K_m of the enzyme is 0.25 mM and it is subject to inhibition by methylxanthines.

4. Corpora lutea which are at the stage of regression contain more Ado-3',5'-*P* phosphodiesterase than those which are collected earlier in the estrous cycle.

5. A preliminary partial purification of the enzyme is described, and its properties are discussed in relation to the properties of Ado-3',5'-*P* phosphodiesterases from other mammalian tissues.

INTRODUCTION

The importance of adenosine 3',5'-cyclic phosphate (Ado-3',5'-*P*) as an intracellular mediator of the action of a number of hormones is now well established[1]. The intracellular level of Ado-3',5'-*P* is dependent upon the activities of at least two enzymes, adenyl cyclase, which catalyses the conversion of ATP into Ado-3',5'-*P* and Ado-3',5'-*P* phosphodiesterase, the only enzyme known to destroy Ado-3',5'-*P* and to terminate its action.

MARSH *et al.*[2] have shown that luteinising hormone can cause increased production of Ado-3',5'-*P* in bovine luteal slices within 15 min of *in vitro* incubation, and Ado-3',5'-*P* stimulates this tissue to produce progesterone[3]. Knowledge is increasing about the way in which some hormones activate adenyl cyclase and hence increase Ado-3',5'-*P* synthesis, but there is little information about the role of Ado-3',5'-*P* phosphodiesterase in terminating the physiological action of Ado-3',5'-*P*.

The properties of luteal adenyl cyclase have been recently described[4]; we wish to report in this paper the presence of Ado-3',5'-*P* phosphodiesterase in corpus luteum and to describe some of the properties of the enzyme present in bovine luteal tissue.

MATERIALS AND METHODS

Ado-3′,5′-*P* and alkaline phosphatase (*Escherichia coli*; type 3) were obtained from Sigma (London) Chemical Co. Ltd. All other reagents were purchased from British Drug Houses Ltd., Poole, and were of Analar grade where possible.

Bovine corpora lutea were obtained from the local abbatoir, human corpora lutea were obtained by courtesy of Professor J. Walker, Department of Obstetrics and Gynaecology, University of Dundee, and rat corpora lutea were obtained as previously described[5] from animals purchased from Scientific Products Farm, Canterbury, Kent. Tissues were homogenised in 0.33 M sucrose in a Virtis "23" homogeniser, and homogenates were centrifuged at 600 × *g* for 30 min to remove debris unless otherwise stated in the text.

Ado-3′,5′-*P* phosphodiesterase activity was determined by the method of WEISS *et al.*[6] in which Ado-3′,5′-*P* (1 μmole) is converted to Ado-5′-*P* at pH 8.0 and in the presence of $3.5 \cdot 10^{-3}$ M Mg^{2+}. Ado-5′-*P* is concurrently hydrolysed by alkaline phosphatase to adenosine and P_i. Tris–HCl buffers (40 mM) were used throughout. P_i was determined by the method of BUELL *et al.*[7] because this procedure was found to be unaffected by the levels of caffeine and theophylline used in the inhibitor studies. Determinations were carried out at least in duplicate, the incubations being in a total volume of 2.0 ml for 20 min at 37° in a shaking water bath. Controls were included to ensure that alkaline phosphatase did not hydrolyse Ado-3′,5′-*P* and to allow corrections to be made for endogenous P_i production in the absence of Ado-3′,5′-*P*.

Activity of phosphodiesterase is expressed as the number of μmoles of P_i produced during 20 min incubation under the above conditions.

Preparation of acetone powders

The method is based on that described by KORZENOVSKY *et al.*[8]. Bovine corpora lutea were minced through a small tissue press (Climpex Ltd., London) into 10 vol. of acetone pre-cooled to −27°. The suspension was stirred for 1 h and allowed to stand overnight at the above temperature before being filtered through sintered glass (pore size 20–30 μm). The filter cake was washed with 100 ml acetone and 100 ml ether, both solvents having been cooled to −15°. The residue was air-dried at 0–5°. Enzyme preparations were obtained from the acetone powder by shaking a suspension of the powder in 40 mM Tris buffer (pH 8.0) for 1.5 h at 0–5°.

Extracts were cleared by centrifugation at 800 × *g* before being assayed for Ado-3′,5′-*P* phosphodiestaerase activity.

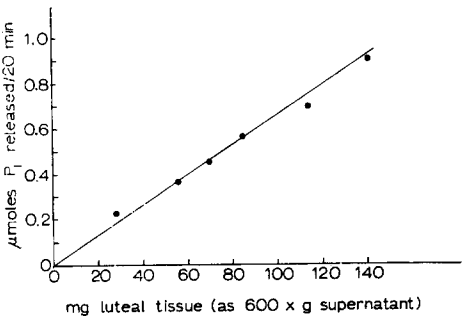
Protein estimations were by micro-Kjeldahl digestion and Markham distillation[9].

RESULTS

Ado-3′,5′-*P* phosphodiesterase activity was observed in homogenates prepared from human, rat and bovine corpora lutea. The 600 × *g* supernatant fractions had the following activities: human, 1.5 units/g; rat (dialysed), 7.8 units/g; bovine, 4.6 units/g.

Properties of the bovine enzyme

The hydrolysis of Ado-3′,5′-*P* increased linearly with increasing amounts of 600 × *g* supernatant present in the incubations (Fig. 1).



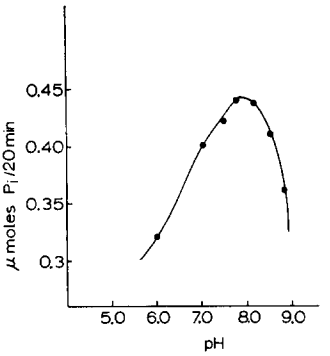


Fig. 1. Graph of luteal Ado-3′,5′-*P* phosphodiesterase activity *vs.* tissue concentration.

Fig. 2. pH optimum of bovine luteal Ado-3′,5′-*P* phosphodiesterase.

Incubations contained tissue extract sufficient to cause not more than 50% hydrolysis of substrate under the standard incubation conditions. The optimum pH for the hydrolysis of Ado-3′,5′-*P* was 8.0 (Fig. 2). The enzyme was relatively unstable to dialysis, 25 % of the activity being lost after 3 h, and 39% being lost after 18 h of dialysis against 0.33 M sucrose solution at 3°.

Fractional centrifugation studies revealed that Ado-3′,5′-*P* phosphodiesterase is mainly located in the 100 000 × *g* supernatant of bovine luteal homogenate (Table I).

The K_m of the enzyme was studied in two ways; firstly, in duplicated samples taken from a single corpus luteum and secondly, in samples of corpora lutea collected at different stages of the estrous cycle. The stage of the cycle at which the tissue was taken for investigation was assessed from the descriptions given by HANSEL[10].

The K_m value of Ado-3′,5′-*P* phosphodiesterase assayed on samples of tissue from a single corpus luteum was constant, but there could be some variation when samples from different corpora lutea were assayed simultaneously (Fig. 3). The range of K_m values was 0.36 to 0.20 mM with the mean value (8 corpora lutea) being 0.25 mM (± standard deviation of 0.05 mM). There was no apparent relationship between the variation of K_m and the stage of the estrous cycle at which the tissue was taken.

Bovine luteal Ado-3′,5′-*P* phosphodiesterase was inhibited by methyl xanthines, in the presence of 10 mM caffeine the enzyme was inhibited to the extent of 75%.

TABLE I

SUBCELLULAR DISTRIBUTION OF BOVINE LUTEAL ADO-3′,5′-*P* PHOSPHODIESTERASE

Fraction	*Activity (%)*
Whole homogenate	100
600 × *g* · 30 min (supernatant)	94
11 500 × *g* · 25 min (supernatant)	88
11 500 × *g* · 25 min (pellet)	5
100 000 × *g* · 40 min (supernatant)	74
100 000 × *g* · 40 min (pellet)	2

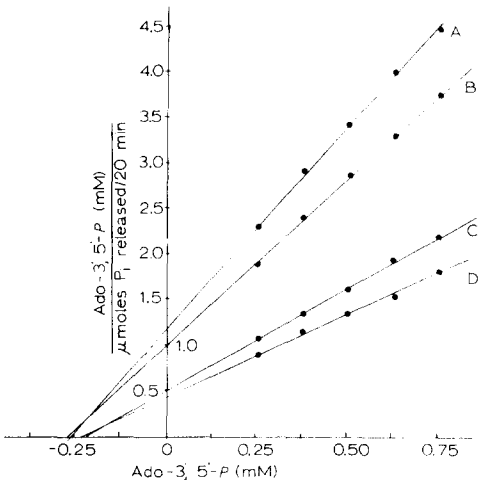


Fig. 3. K_m determinations on samples removed from bovine corpora lutea at various stages of the estrous cycle. The plot is of $[S]/v$ *vs.* $[S]$. A, estimated stage of cycle, 5 days; B, estimated stage of cycle, 5 days. C, estimated stage of cycle, 15 days; D, estimated stage of cycle, 20 days. K_m values (mM): A, 0.26; B, 0.28; C, 0.23; D, 0.24.

Preliminary results with 600 × *g* supernatant as source of enzyme indicate that this inhibition is of the mixed type. Bovine luteal Ado-3′,5′-*P* phosphodiesterase was activated maximally (37% activation) by imidazole at a concentration of 0.04 M.

The amount of Ado-3′,5′-*P* phosphodiesterase activity increased throughout the estrous cycle; Table II shows that the activity of this enzyme is highest in those corpora lutea which are at the stage of regression. The values of v_{max} obtained during kinetic analyses for K_m show a similar trend when related to the stage of the cycle at which the tissue was removed (Fig. 4).

Purification of Ado-3′,5′-P phosphodiesterase from bovine corpus luteum

Initial attempts at fractionation of luteal homogenates by salting out with ammonium sulphate were unsuccessful. The protein precipitates would not centrifuge

TABLE II

ADO-3′,5′-*P* PHOSPHODIESTERASE ACTIVITY OF BOVINE CORPORA LUTEA AT TIMES DURING THE ESTROUS CYCLE

Data	*Corpus luteum colour*				
	Brown	*Brown*	*Yellow-orange*	*Red-orange*	*Red-orange*
Estimated day of cycle	5	5	15	20	20
Total enzyme units per corpus luteum	3.08	3.95	13.8	25.0	27.0
Enzyme units/g tissue	2.88	2.87	3.90	5.90	5.88

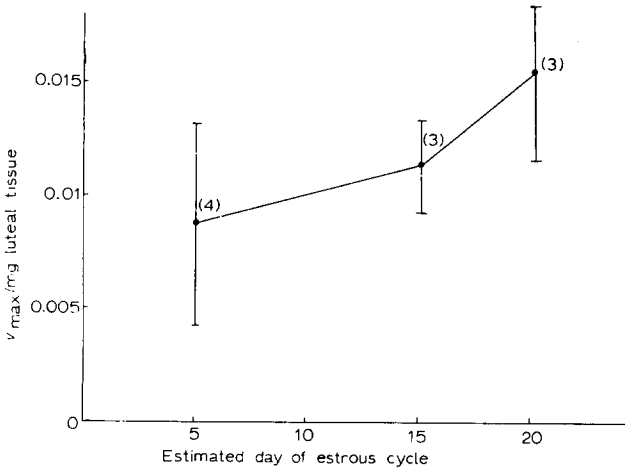


Fig. 4. v_{max}/mg luteal tissue plotted *vs.* day of the estrous cycle at which the ovary was removed. v_{max} was calculated in a series of experiments similar to, and including, that shown in Fig. 3, and shown as the mean ± standard deviation. Figures in parentheses indicate the number of observations.

down cleanly because of the very high lipid content of the luteal tissue. In order to remove lipid acetone powders of bovine corpora lutea were prepared.

These were active with respect to hydrolysis of Ado-3′,5′-*P* when extracted by shaking with Tris buffer. Acetone powder extracts, however, did have one drawback; their high inorganic phosphate content gave rather high "blanks" in the phosphate analysis with which the enzyme assay is terminated. 76% of the phosphodiesterase activity and 8.6% of the initial protein content was precipitated out of the 100 000 × *g* supernatant of luteal acetone powder extract by raising the percentage saturation with $(NH_4)_2SO_4$ from 40 to 50. The specific activity of the enzyme was raised approximately 10 times relative to that of the 100 000 × *g* supernatant by this procedure

DISCUSSION

The Ado-3′,5′-*P* phosphodiesterase of bovine corpus luteum is located in the 100 000 × *g* supernatant fraction (Table I) in agreement with the findings for dog heart[11], rabbit brain[12] and rat pineal gland[13]. In some other tissues the enzyme has also been reported to occur in particulate fractions[14–16].

The pH optimum for the luteal enzyme (pH 8.0) (Fig. 2) is identical with that shown for Ado-3′,5′-*P* phosphodiesterase from rat kidney[17] and rat brain[15] and is close to that shown for the beef heart enzyme[14]. However, NAIR[11] has shown that the pH optimum of the dog heart enzyme is 8.5–9.2.

The K_m of luteal Ado-3′,5′-*P* phosphodiesterase, assayed in 600 × *g* supernatant of crude homogenate, was variable over a relatively small range. The variability did not appear in samples taken from a single corpus luteum, suggesting that it is due to inter-animal differences rather than imprecise technique. The variation of K_m was not apparently related to the stage of the estrous cycle at which the tissue was collected. Variability of K_m has been reported for brain Ado-3′,5′-*P* phosphodieste-

rase[15], the values lying between 0.1 and 0.3 mM. In our experiments the K_m values lay within the range 0.20–0.36 mM, with a mean value of 0.25 mM. Other estimates of K_m for Ado-3′,5′-*P* phosphodiesterase have been 0.49 mM (dog heart)[11], 0.29 mM (rat kidney)[17] and 0.06–0.1 mM (beef heart)[14].

Caffeine and theophylline inhibit luteal Ado-3′,5′-*P* phosphodiesterase, the inhibition seeming to have mixed characteristics. Competitive inhibition has been shown for the Ado-3′,5′-*P* phosphodiesterases of some other mammalian tissues[14,15,17] but NAIR[11] claims non-competitive kinetics for the dog heart enzyme. The inhibition characteristics are to be investigated more fully on Ado-3′,5′-*P* phosphodiesterase purified according to the procedure described in this paper. The observed activation of luteal Ado-3′,5′-*P* phosphodiesterase by imidazole is in line with other reports[11,14,15] The necessity for work with more purified preparations of Ado-3′,5′-*P* phosphodiesterase has been indicated by the observation by CHEUNG[18] of an activator of beef brain Ado-3′,5′-*P* phosphodiesterase in its crude homogenates.

We believe that our observations that luteal Ado-3′,5′-*P* phosphodiesterase activity increases in activity throughout the estrous cycle is of considerable importance (Table II and Fig. 4). The increase is seen, not only on a total enzyme/corpus luteum basis, but also on an enzyme activity/g tissue basis. Ado-3′,5′-*P* is known to stimulate progesterone synthesis in corpus luteum[3], and it is of interest to speculate that increasing amounts of the enzyme, which bring about the destruction of Ado-3′,5′-*P* may be related to the observed inability of bovine corpora lutea taken on or after day 19 of the cycle to produce progesterone (discussed in ref. 19).

It is possible that increasing amounts of Ado-3′,5′-*P* phosphodiesterase in corpus luteum may be one factor in the ultimate cessation of steroid synthesis and secretion in this organ.

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