

CYCLIC ADENOSINE 3', 5' – MONOPHOSPHATE AND LUTEINIZING HORMONE STIMULATED PROTEIN KINASE FROM BOVINE CORPUS LUTEUM: EVIDENCE FOR ACTIVATION THROUGH SEPARATE MECHANISMS*

Salman AZHAR** and K. M. J. MENON

*Reproductive Endocrinology Program, Departments of Obstetrics and Gynecology and Biological Chemistry,
The University of Michigan Medical Center, Ann Arbor, Michigan 48104, USA*

Received 18 November 1974

1. Introduction

Protein kinases catalyze the transfer of the terminal phosphate of ATP to a variety of acceptor proteins [1]. It has been demonstrated that cyclic adenosine 3', 5'-monophosphate (cyclic AMP) activates protein kinase (holoenzyme, RC) by forming a complex with the regulatory subunit (R) to release the catalytic subunit (C) in the active state [2,3].

In a previous communication from this laboratory the purification and properties of two protein kinases (KI and KII) from bovine corpus luteum has been described [4]. In addition to stimulation by cyclic AMP, KII was also stimulated directly by luteinizing hormone (LH). From these studies it was inferred that LH may have a direct control on the activity of KII and that this effect is independent of cyclic AMP. The present investigation was undertaken to compare the effect of LH with that of cyclic AMP on the stimulation of KII from bovine corpus luteum. Evidence is presented to suggest that cyclic AMP stimulates the activity of KII by binding to the regulatory subunit thereby releasing the activated catalytic subunit whereas LH acts without dissociating the regulatory-catalytic subunit complex.

2. Methods

Protein kinase (KII) was purified from bovine corpus luteum according to previously published procedure [4]. [γ - 32 P]ATP was prepared by the procedure of Glynn and Chappell [5]. Protein kinase activity was determined by the procedure of Kuo et al [6] with minor modifications [4]. The incubations were performed in 0.2 ml reaction mixture containing: 1 nmol [γ - 32 P]ATP (1.5×10^5 cpm); 2 μ mol KF; 2 μ mol MgCl₂; 0.2 mg calf thymus mixed histone (Type IIA); 10 μ mol of α -glycerophosphate buffer pH 6.0; and where required, 5 μ g LH or 2 nmol cyclic AMP. After incubation at 30°C for 15 min, 2 ml of 10% trichloroacetic acid (w/v) and 0.2 ml of 0.63% bovine serum albumin were added. The tubes were then processed for radioactivity determinations as described earlier [4]. Cyclic AMP binding activity was assayed according to Gilman [7]. Sucrose density gradient centrifugations were carried out according to Martin and Ames [9] with catalase (11.6S, mol. wt 232 000), glyceraldehyde 3-phosphate dehydrogenase (7.7S, mol. wt 140 000) and horse liver alcohol dehydrogenase (5.4S, mol. wt 84 000) as internal markers [10]. Separation of catalytic and regulatory subunits by cyclic AMP was achieved as given under fig.2.

3. Results and discussion

The effect of LH and cyclic AMP on the phosphorylation of histone by KII is shown in table 1. Both LH

* Supported by NIH grant HD06656.

** Postdoctoral Fellow of the Population Council.

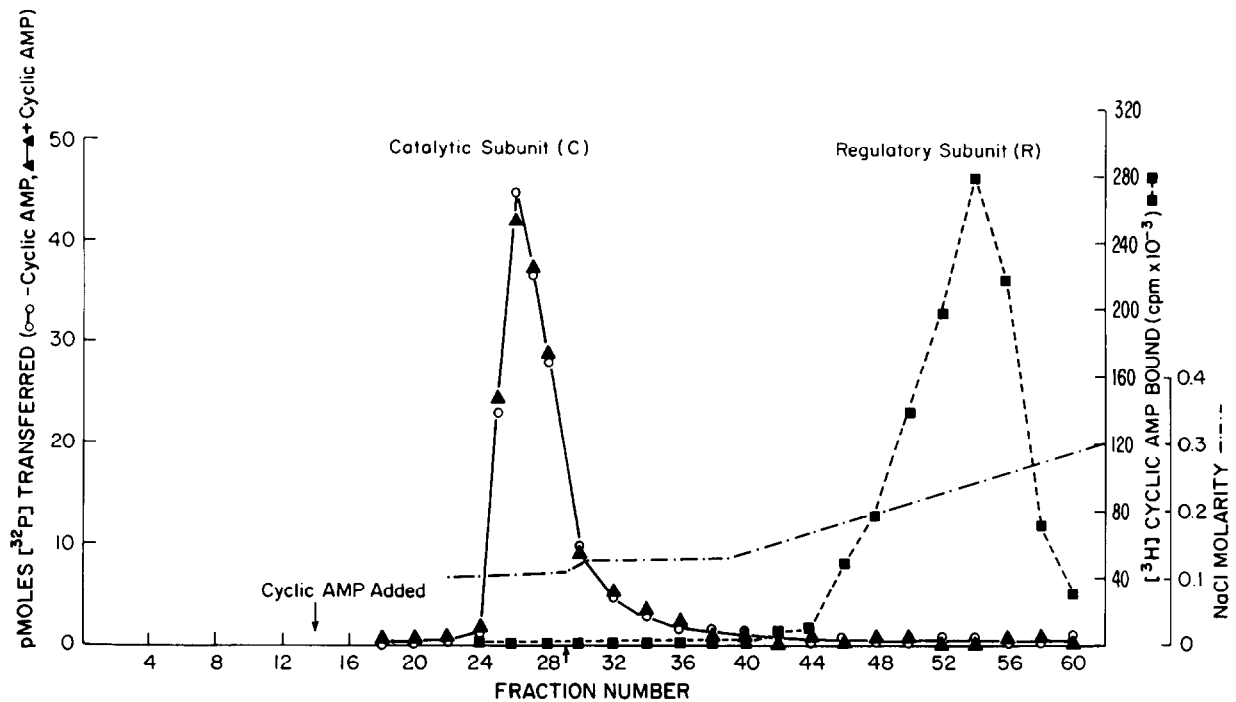


Fig.1. Separation of the cyclic AMP binding (R) and protein kinase subunits (C) by cyclic AMP-DEAE cellulose column chromatography. Protein kinase II (10 mg protein) was adsorbed to DE-23 (2 x 15 cm) and washed with (i) 10 ml Tris-HCl buffer pH 7.4 (10 mM); (ii) 10 ml of Tris-0.05 M NaCl; (iii) 50 ml of Tris-0.075 M NaCl-10 μ M cyclic AMP; (iv) 50 ml of Tris-M NaCl and finally with a linear gradient from 0.15 M to 0.4 M NaCl (70 ml each). The fractions were dialyzed for 15 hr against three changes of Tris-HCl buffer (0.01 M) containing 10% glycerol and 0.05 mM 2-mercaptoethanol. The active fractions were concentrated by ultrafiltration (Amicon diaflo ultra-filter P MIO).

and cyclic AMP stimulated histone phosphorylation. The data obtained with only two concentrations of LH and cyclic AMP are presented. In order to determine

Table 1
Effect of cyclic AMP and LH on the phosphorylation of histone by KII from bovine corpus luteum

Additions	pmol $\times 10^{-2}$ [32 P]phosphate transferred 15 min/mg protein
None	17.0
Cyclic AMP (1 μ M)	63.0
Cyclic AMP (5 μ M)	56.0
LH-NIH-B7 (5 μ g)	26.0
LH-NIH-B7 (10 μ g)	30.0

The enzyme activity was assayed as described under Materials and methods. 20 μ g purified KII was used per incubation mixture.

whether the mode of action of the LH is similar to that of cyclic AMP, that is, the dissociation of the holoenzyme into catalytic and regulatory subunits, the following experiments were performed. KII was incubated in the presence of 1 μ M cyclic AMP or 20 μ g LH and applied to sucrose gradient (5-20% w/v) containing the same concentration of these substances. A control contained neither cyclic AMP nor LH. Results presented in fig.2 show that, as expected, cyclic AMP caused the dissociation of catalytic and regulatory subunits of KII, while LH showed no effect. As separation of subunits was not complete on sucrose gradient, we have separated the two activities free from each other by cyclic AMP-DEAE cellulose column chromatography. The activity of the catalytic subunit isolated by this procedure was independent of cyclic AMP and the regulatory subunit was devoid of kinase activity (fig.1). The results presented in fig.3. shows

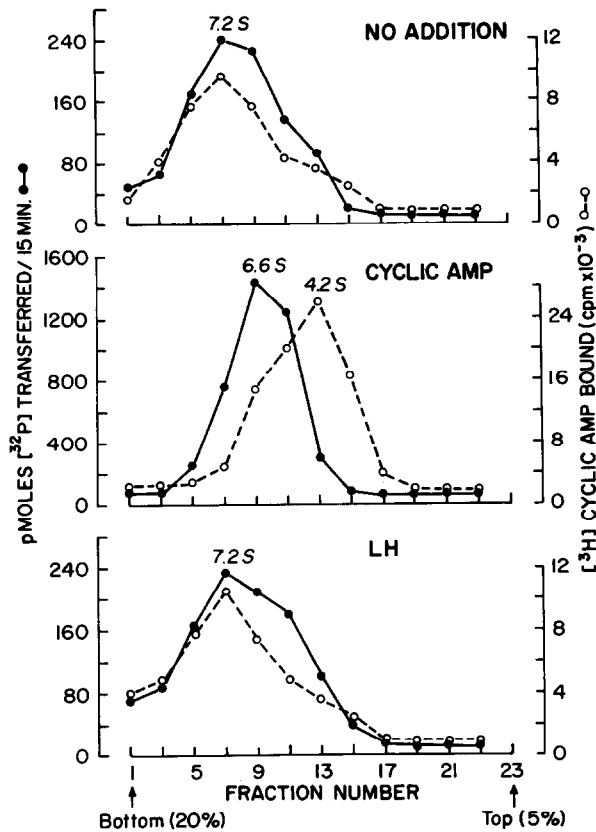


Fig.2. Sucrose density gradient centrifugation (5–20%) of protein kinase II (2 mg) in a medium containing 200 mM Tris-HCl, pH 7.4, 0.1 mM EDTA and 1 mM dithiothreitol in the absence and presence of 1 μ M cyclic AMP or LH (20 μ g). Centrifugation was carried out at 189 000 g for 18 hr. Cyclic AMP binding activity and protein kinase activity were determined as described under 'Materials and methods'.

the inhibition of catalytic subunit by increasing concentrations of the regulatory subunit. Cyclic AMP, when present in the medium prevented the inhibition (fig.3, table 2) by regulatory subunit. Regulatory subunit showed a mol. wt of 84 000 on filtration through Sephadex G-200 column (2.5 \times 38 cm) whereas the mol. wt of the catalytic subunit was 170 000. The relatively large mol. wt of the catalytic subunit may be due to dimerization [11]. Under similar conditions, holoenzyme showed a mol. wt of 160 000.

The results presented suggest that cyclic AMP dependent protein kinase II in bovine corpus luteum exists

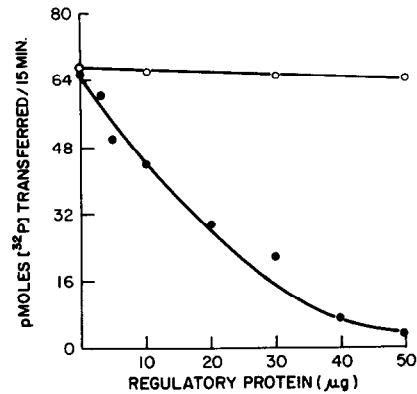


Fig.3. Effect of 'R' protein and cyclic AMP on protein and cyclic AMP on protein kinase subunit 'C'. The 'R' protein was added to the standard reaction mixture as indicated, which contained 3.1 μ g 'C' subunit. Cyclic AMP (4 μ M) was added where indicated. Open circles indicate the catalytic activity of a fixed concentration of 'R' protein and 4 \times 10⁻⁶ M cyclic AMP. The closed circles represent the catalytic activity of 'C' subunit in the presence of increasing concentration of 'R' protein in the absence of cyclic AMP.

in less active form, due to its association with 'R' protein, and that cyclic AMP binds to 'R' protein and then releases the 'C' subunit to exhibit enhanced activity. Upon dissociation by cyclic AMP bovine corpus luteum KII showed only a single peak for cyclic AMP binding and phosphotransferase activities.

Table 2
Properties of catalytic (C) protein and regulatory (R) protein from bovine corpus luteum KII

Additions	pmol [³² P]phosphate transferred/15 min
Experiment 1	
C-protein (2.4 μ g)	36
C-protein (2.4 μ g) + Cyclic AMP (4 μ M)	38
R-protein (10 μ g)	0
Experiment 2	
C-protein (3.7 μ g)	67
C-protein (3.7 μ g) + R-protein (10 μ g)	44
C-protein (3.7 μ g) + R-protein (10 μ g) + cyclic AMP (4 μ M)	66

The enzyme activity was assayed as described under Materials and methods.

The evidence presented in this paper suggests that cyclic AMP stimulation of KII follows the same mechanism (table 2, fig.3) as has been reported in other systems [2,3,8,11-13], however, stimulation of KII by LH does not follow the same mechanism. It is possible that LH could cause a greater affinity of histone for KII or it could activate catalytic subunit directly without affecting catalytic-regulatory subunit complex. These studies also suggest that the stimulatory effect exhibited by LH is not due to the contamination of LH preparations with cyclic AMP. Present studies thus suggest that the mechanism of direct stimulation of protein kinase II by LH is different from that observed with cyclic AMP.

Acknowledgements

We wish to thank Mrs Kathie Nosal for expert technical assistance and Mrs Cindy Giraud and Mrs Joanne Robinson for secretarial help. NIH-LH-B7 was a generous gift from the Hormone Distribution Program, National Institutes of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health.

References

- [1] Greengard, P. and Kuo, J. F. (1970) In: Role of Cyclic AMP in Cell Function (Greengard, P. and Costa, E., eds.) p. 287, Raven Press, New York.
- [2] Krebs, E. G. (1972) In: Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R., eds.) Vol. 5, p. 99, Academic Press, New York.
- [3] Bitensky, M. W. and Gorman, R. E. (1973) *Pogr. Biophys. Mol. Biol.* 26, 411.
- [4] Menon, K. M. J. (1973) *J. Biol. Chem.* 248, 494.
- [5] Glynn, I. M. and Chappell, J. B. (1964) *Biochem. J.* 90, 147.
- [6] Kuo, J. F. and Greengard, P. (1969) *Proc. Natl. Acad. Sci. U.S.* 64, 1349.
- [7] Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 305.
- [8] Sanborn, B. M., Bhalla, R. C. and Korenman, S. G. (1973) *J. Biol. Chem.* 248, 3593.
- [9] Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem.* 236, 1372.
- [10] Miyamoto, E., Petzold, G. L., Kuo, J. F. and Greengard, P. (1973) *J. Biol. Chem.* 248, 179.
- [11] Gill, G. N. and Garren, L. D. (1970) *Biochem. Biophys. Res. Comm.* 39, 335.
- [12] Garren, L. D., Gill, G. N., Masui, H. and Walton, G. M. (1971) *Recent Pogr. Hormone Res.* 27, 433.
- [13] Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1971) *J. Biol. Chem.* 246, 1968.