STIMULATION BY LH OF CYTOSOLIC PROTEIN PHOSPHORYLATION IN BOVINE LUTEAL CELLS

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1. Introduction

It is now well established that LH exerts its stimulatory action on steroidogenesis in luteal tissue by increasing the intracellular level of cyclic AMP (cAMP) [1]. Moreover the cAMP dependent protein kinase activity has been shown to increase in the cytosol of bovine luteal tissue incubated in the presence of LH [2]. Two points suggest the implication of this type of enzyme in the stimulation of steroidogenesis. On one hand, the enzyme has been shown to mediate in other tissues several hormonal effects involving cAMP such as the glycogenolytic effect of epinephrine and glucagon in liver [3], epinephrine in muscle [4] and the lipolytic effect of epinephrine in adipose tissue [5]. On the other hand, two partially purified luteal enzymes, possibly involved in the steroidogenesis regulation by LH, have been shown to be slightly stimulated by incubation in the presence of cAMP-dependent protein kinase. These enzymes are a "reconstituted" cholesterol side chain cleavage enzyme [6] and a sterol ester hydrolase enzyme [7]. In vitro hormonal induction of intracellular protein phosphorylation has been demonstrated for a small number of hormones: glucagon on hepatocytes [8,9] luteinizing hormone on Leydig cells [10], and norepinephrine on adipocytes [11].

The present work was an attempt to extend this latter type of findings to luteal cells stimulated in vitro by LH and to bring some further argument in favor of the implication of the cAMP-dependent protein kinase in the mechanism of the hormone action. Using a suspension of small bovine luteal cells which are known to be highly sensitive to LH [12] we have been able to demonstrate a dose-dependent stimulatory effect of this hormone on ^{32}P incorporation into several cytosolic proteins and a correlation between the stimulation of protein phosphorylation and the stimulation of steroidogenesis.

2. Materials and methods

2.1. *Chemicals*

Histone type IIA, egg white trypsin inhibitor, ATP, cAMP, dibutyryl cAMP, theophylline, bovine serum albumin were obtained from Sigma. LH was a gift from the NIH. [^{32}P]phosphoric acid, [γ-^{32}P]ATP (0.5–3 Ci/mmol) cAMP assay kit were obtained from Amersham. Acrylamide, bisacrylamide, Coomassie blue were purchased from Eastman Kodak. X-ray films (Xomat R) were from Kodak and the intensifying screens (Cronex lightning plus) from Dupont. All other chemicals were from Merck.

2.2. *Methods*

2.2.1. Preparation of the small luteal cell suspension

Corpora lutea from from 60 to 100 day pregnant cows were collected at the slaughterhouse and the luteal cells were obtained as previously described [12] by enzymatic dissociation of the tissue and separation of the small luteal cells (18 μm average diameter) by sedimentation velocity at unit gravity.

2.2.2. Incubations

The small luteal cell suspension (0.5 to 1 · 10^6 cells by beaker) was preincubated for 1 h under O_2/CO_2 (95/5) at 37°C in 1 ml of KRB, pH 7.3 containing 0.1% BSA, 0.1% trypsin inhibitor, 0.2% glucose, 0.5 mM theophylline, 20 mM Hepes and devoided of phosphate (KCl substituted to KH_2PO_4).

After centrifugation and decantation, the cells were resuspended in 450 μl of the same fresh buffer containing 10^{-5} M actinomycin D*, an inhibitor of RNA synthesis, and 50 μCi of [^{32}P]phosphoric acid-HCl neutralized with 0.1 N NaOH. The cells were preincubated in this medium for 1 h and incubated for another hour in a final volume of 500 μl in the absence or presence of varying doses of bovine LH or 5 mM DB cAMP. For cAMP and protein kinase activity measurements, ^{32}P was replaced by ^{31}P. Incubation was stopped by addition of 1 ml of ice-cold KRB. The progesterone content was measured on small aliquots. After centrifugation the cells were resuspended in 250 μl of homogeneization buffer: 10 mM Tris-HCL, pH 7.4, containing 10% glycerol, 6 mM β-mercaptoethanol, 2 mM EDTA and 100 mM NaF and frozen. For protein kinase activity determination, NaF was omitted, 0.15 M NaCl and 8 mM theophylline were added.

2.2.3. Preparation of the cytosolic fractions

After thawing, the cell suspensions were sonicated for 20 s and after removal of the top lipid layer, the 105 000 × *g* supernatants were collected and small aliquots taken for protein content estimation according to Lowry [13].

2.2.4. SDS polyacrylamide slab gel electrophoresis

Cytosolic proteins were fractionated by electrophoresis on 4.5% and 10% (w/v) discontinuous sodium dodecyl sulfate polyacrylamide slab gels as described by Laemmli [14]. All proteins with an apparent molecular weight between 130 000 and 25 000 were fractionated.

2.2.5. Autoradiography

After electrophoresis the gels were soaked in 50% trichloroacetic acid (TCA) during 30 min, stained overnight in 0.05% Coomassie blue, 12.5% TCA and 0.15 M KCl, then destained in 10% TCA and hot 5% TCA according to Avruch et al. [8]. The gels were returned to a solution of 10% acetic acid and 1% glycerol for 5 h.

The dried gels were exposed to Kodak Xomat R films with Dupont Cronex lightning plus intensifying screen during 2 to 8 days. The radioautographs were scanned at 540 nm.

2.2.6. Progesterone, cAMP and protein kinase assay

Progesterone was measured by radioimmunoassay as previously described [12]. The cAMP released by cells in the incubation medium was assayed by a slightly modified Gilman procedure [15].

The endogenous protein kinase activity was measured as previously described [2] with the following modifications: the ATP and histone concentrations were respectively 0.05 mM (125 cpm/pmol) and 1 mg/ml. Total protein kinase activity was measured in the presence of 5 μM exogenous cAMP.

3. Results and discussion

In five successive incubations in the absence of LH, the distribution of ^{32}P among the fractionated proteins was similar and showed about 10 major phosphorylated protein bands. The gonadotropin LH consistently stimulated the ^{32}P incorporation into at least five luteal cytosolic proteins of apparent molecular weight 120 000, 100 000, 95 000, 85 000 and 65 000. The degree of phosphorylation varied slightly from one experiment to another. In every case the maximum LH effect was observed for the two proteins of molecular weight 120 000 (A) and 85 000 (D). Fig.1 shows the results of a typical experiment. DB cAMP mimicked the LH action (table 1).

The time course of phosphorylation was studied for the two proteins A and D. A significant increase in ^{32}P content was already measurable after 5 min of incubation with LH (table 2). The LH induced stimulation of progesterone synthesis was also clearly measurable after 5 min of incubation.

A comparison of ^{32}P incorporation into proteins A and D, cytosolic protein kinase activity, cAMP production and progesterone synthesis was then undertaken after 1 h incubation in the absence or presence of 3 and 30 ng/ml of LH. The results, given in table 3, indicate that the 3 ng/ml dose, which has previously been found to produce a half maximal stimulation of progesterone synthesis [12] clearly stimulated ^{32}P incorporation into proteins A and D. The extent of this stimulation was more than 50% of that observed after incubation with 30 ng/ml of LH, a dose that itself had been shown, in a parallel study (unpublished results), to produce a nearly maximal

* This concentration of actinomycin D has been shown not to influence a maximal stimulation of progesterone synthesis in our experimental conditions (unpublished results).

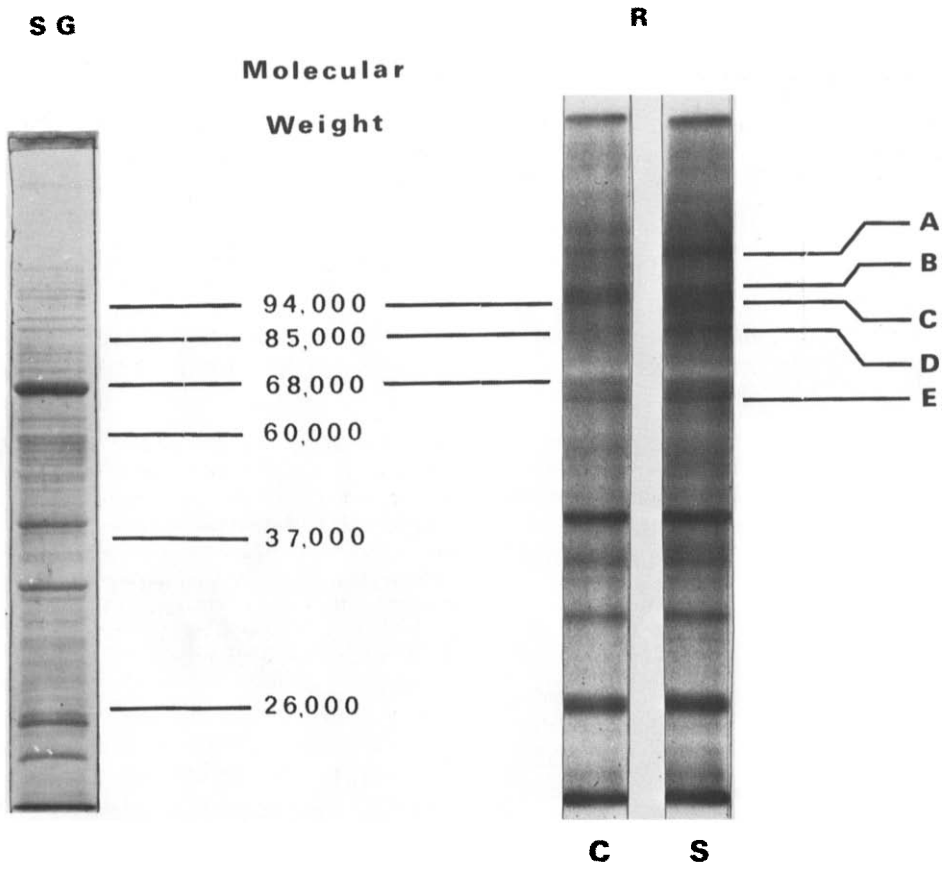

Fig.1. Effect of LH on phosphorylation of cytosolic proteins in intact small luteal cells. Luteal cells were incubated for 1 h in the absence (C) or in the presence (S) of 30 ng/ml LH. Cytosolic proteins were separated by polyacrylamide SDS gel electrophoresis. Radioautographs of the dehydrated gel (R) are shown side by side with a representative stained gel (SG). Molecular weights indicated on the right hand side of the SG were evaluated by the use of the following marker proteins: phosphorylase b (94 000) transferrin (85 000) human albumin (68 000) catalase (60 000) yeast alcohol dehydrogenase (37 000) and chymotrypsinogen A (26 000). A marked stimulation was observed in the five following proteins: A (120 000) B (100 000) C (95 000) D (85 000) E (65 000).

stimulation of ^{32}P incorporation. Hence the amount of ^{32}P incorporated with different doses of LH approximately followed the increase in progesterone synthesis.

In contrast with these results, protein kinase activation was not detectable for 3 ng/ml of LH and reached only approx. 15% of its maximal level, evaluated by activity measurement in the presence of 5 μM exogenous cAMP, for 30 ng/ml of LH. The cyclic AMP concentration itself was only slightly stimulated by 3 ng/ml of LH and the large increase observed with 30 ng/ml was still less than a third of the maximum stimulation observed simultaneously with a saturating dose of LH (unpublished data).

Such discrepancies at low hormone concentration between cAMP rise, protein kinase activation and steroidogenesis have been reported for other tissues, particularly for testicular interstitial cells stimulated by hCG [16]. It was of interest to notice that the small increment of cAMP which seemed to be related to a half maximal stimulation of steroidogenesis and which was produced by a physiological concentration of LH was correlated with a half maximal stimulation of protein phosphorylation.

These preliminary data demonstrate an in vitro stimulation by LH of endogenous cytosolic protein phosphorylation which was closely correlated with the stimulation of steroidogenesis. They confirm that

Table 1
Stimulation by LH and DB cAMP of cytosolic protein phosphorylation in luteal cells

Protein band	Molecular weight ($\cdot 10^{-3}$)	Increase in ^{32}P incorporation	
		LH	DB cAMP
A	120	148 ± 39	138 ± 40
B	100	78 ± 18	109 ± 16
C	95	76 ± 28	58 ± 13
D	85	151 ± 29	108 ± 7
E	65	78 ± 17	58 ± 11

Luteal cells were incubated for 1 h in the absence (C) or in the presence (S) of 30 ng/ml LH or 5 mM DB cAMP. Autoradiographs were scanned by densitometry and the ^{32}P incorporation in the different proteins was evaluated by measurement of the respective peak heights. Stimulation by LH or DB cAMP was expressed as percent of control calculated as follows: $\frac{S - C}{C} \cdot 100$. Values are means ± SEM of 5 experiments

cAMP could exert its stimulatory action on steroidogenesis in luteal tissue by regulating the phosphorylation of one or several endogenous cytosolic proteins. The nature of these phosphorylated substrates remains at the present time unknown.

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Table 2
Cytosolic protein phosphorylation and "de novo" progesterone synthesis for various times of exposure to LH

Incubation time		Protein phosphorylation (arbitrary units)		"de novo" Progesterone Synthesis (ng/10^5 cells)
		A	D	
5 min	C	4 ± 0.8	4.1 ± 0.9	53 ± 9
	S	11.3 ± 1.7	9.1 ± 1.7	144 ± 9
20 min	C	3.9 ± 0.1	4.3 ± 0.1	67 ± 4
	S	10.2 ± 0.1	8.4 ± 0.1	255 ± 11
60 min	C	6.9 ± 1.1	7.8 ± 1.3	88 ± 11
	S	12 ± 1	10.9 ± 1.1	335 ± 44

Small luteal cells were incubated at 5, 20 and 60 min in the absence (C) or in the presence (S) of 300 ng/ml LH. The ^{32}P incorporation was evaluated for cytosolic proteins 120 000 and 85 000 by densitometric scanning of the radioautograph and measurement of the respective peak heights. The results are expressed in arbitrary units. Each value is the mean of two measurements from two separate electrophoretic gels. The "de novo" progesterone synthesis was evaluated by quadruplicate measurements

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Table 3
Effect of two LH concentrations on cAMP rise, protein kinase activation, protein phosphorylation and "de novo" progesterone synthesis

LH concentration (ng/ml)	cAMP pmol/mg prot.	Protein kinase activity pmol ^{32}P inc./min/mg prot.	Protein phosphorylation (arbitrary units)		"de novo" progesterone synthesis (ng/10^5 cells)
			A	D	
C	12 ± 1	110 ± 6	1.8 ± 0.2	1.9 ± 0.1	1.3 ± 0.1
3	17 ± 2	112 ± 11	5.2 ± 0.3	5.3 ± 0.1	19.8 ± 1.9
30	66 ± 16	127 ± 6	7.9 ± 0.9	6.9 ± 0.7	46.0 ± 1.4

Luteal cells were incubated for 1 h in the absence (C) or in the presence of 3 ng/ml and 30 ng/ml LH. The cAMP released by cells in the incubation medium was expressed in pmol/mg of protein. Each value is the mean (± SD) of quadruplicate measurements. The cytosolic protein kinase activity was expressed in pmol of ^{32}P incorporated/min/mg of protein. Each value is the mean (± SD) of triplicate measurements. The ^{32}P incorporation, expressed as in table 2, was measured for cytosolic proteins 120 000 and 85 000 on two radioautographs. The "de novo" progesterone synthesis was evaluated by quadruplicate measurements

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