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Ca^{2+}-DEPENDENT PHOSPHORYLATION OF RAT OVARY PROTEINS

Evelyn T. Maizels and Richard A. Jungmann

Department of Molecular Biology
Northwestern University Medical School
Chicago, Il. 60611

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A 105,000 x g supernatant fraction from prepubertal rat ovaries was incubated in the presence of [γ-^{32}P]ATP. Phosphorylated proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and identified by autoradiography. Inclusion of Ca^{2+} in the phosphorylation reaction promoted a selective ^{32}P incorporation into two proteins of M_r = 95,000 and 50,000. Inclusion of chlorpromazine with Ca^{2+} blocked the Ca^{2+}-stimulated increase of ^{32}P incorporation. Our results demonstrate the presence of Ca^{2+}-stimulated protein phosphorylation system capable of recognizing endogenous substrate proteins in the prepubertal rat ovary.

Ca^{2+} has been recognized to play a key role in the regulation of cellular metabolic processes (1). Among the diverse effects, calcium ions regulate trophic hormone-induced steroidogenesis and steroid release (2-11). Additionally, various other trophic hormone-induced metabolic changes are Ca^{2+}-dependent, e.g. the luteinizing hormone-mediated induction of ornithine decarboxylase in differentiated porcine granulosa cells (12), the gonadotropin-induced increase of proteoglycan production by bovine granulosa cells (13), and phospholipid turnover in adrenocortical cells (14, 15). The mechanism by which Ca^{2+} participates in these processes remains to be elucidated.

One of the conceivable mechanisms of action through which calcium ions achieve their physiological effects could involve the regulation of intracellular phosphorylating activities and phosphory-

The abbreviations used are: EGTA, ethylene glycol bis (aminoethyl-ether)-N, N, N', N'-tetraacetate; SDS, sodium dodecyl sulfate.

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lative modification of intracellular proteins. Phosphorylation of proteins has been recognized as a major post-translational regulatory mechanism leading to a functional modulation of enzyme activities (16) and participating in the control of eucaryotic gene expression (17). Phosphorylation of selective proteins accompanies trophic hormone stimulation of steroidogenic tissues (18-24). There is convincing evidence that cyclic AMP-dependent phosphorylation occurs as a consequence of trophic hormone binding to target cell receptors, adenylate cyclase activation, and activation of cyclic AMP-dependent protein kinases (25-27). In recent years, it has been recognized that Ca^{2+}-dependent phosphorylation co-regulates a variety of metabolic processes in conjunction with cyclic AMP-dependent phosphorylation (28-31). In view of the regulatory role of Ca^{2+} and the conceivable involvement of Ca^{2+}-dependent phosphorylation in these metabolic processes, we have examined rat ovarian tissue for the existence of a phosphorylation system able to recognize endogenous substrates in response to calcium ion stimulation.

MATERIALS AND METHODS

Biochemicals were obtained from Sigma Chemical Company, St. Louis, MO. Electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, CA. [γ-^{32}P]ATP (1000-3000 Ci/mmol) was obtained from New England Nuclear, Boston, MA. and diluted with cold ATP for use. Sephadex G-25 was obtained from Pharmacia Fine Chemicals. Piscataway, N.J.

Ovaries were excised from 26 day old Charles River Sprague Dawley female virgin rats. Tissue was homogenized in 10 volumes of 10 mM Tris, pH 7.8, 10 mM EGTA. The homogenate was centrifuged at 105,000 x g for 60 min. The supernatant fraction was collected and subjected to gel filtration on Sephadex G-25. Elution was carried out with 10 mM Tris, pH 7.8, 0.1 mM EGTA. The eluted void volume was used as the protein source for the *in vitro* phosphorylation reactions. Protein concentration was determined by the method of Lowry (32) with bovine serum albumin as standard.

In vitro phosphorylation was carried out according to O'Callaghan *et. al.* (33) with the following modifications. A 0.2 ml final volume contained 50 mM α-glycerol phosphate, pH 7.0, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 nmol [γ-^{32}P]ATP (5 μCi)and 100 μg of protein. In addition to the above reagents, Ca^{2+}-containing samples contained 0.05 mM EGTA and 0.25 mM $CaCl_2$. The EGTA control sample contained 0.3 mM EGTA and no calcium additions. Chlorpromazine

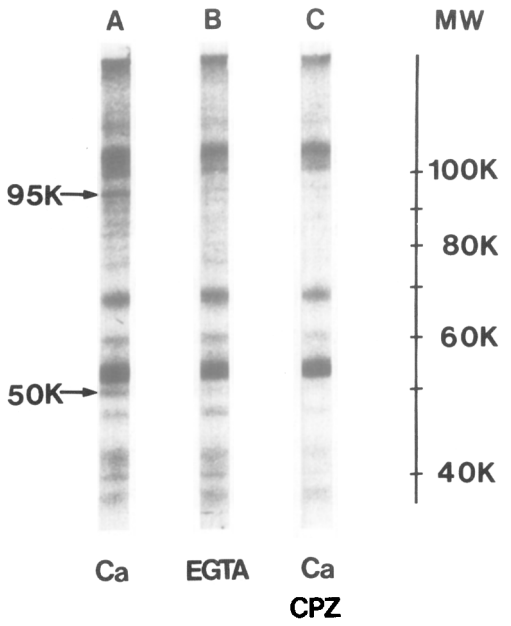

Figure 1. Autoradiogram showing ^{32}P incorporation into rat ovary protein. Rat ovary cytosol was incubated with [γ-^{32}P]ATP as described in Materials and Methods. Proteins were separated by SDS polyacrylamide gel electrophoresis and ^{32}P-labeled proteins identified by autoradiography. Incubations were carried out with: lane A, 0.2 mM free Ca^{2+}; lane B, 0.3 mM EGTA; lane C, 0.2 mM free Ca^{2+} and 0.2 mM chlorpromazine. The molecular weight (MW) range is indicated on the right; 95K and 50K arrows indicate the positions of rat ovary phosphoproteins.

was used at a concentration of 0.2 mM. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP and incubated at 37°C for 60 seconds. The reaction was terminated by the addition of 0.1 ml of stop solution (3% SDS, 3% β-mercaptoethanol, 150 mM Tris, pH 6.8, 2.4 mM EDTA, 30% glycerol and 0.05% bromophenol blue) and heating at 100°C for 5 minutes.

Discontinuous SDS polyacrylamide slab gel electrophoresis was carried out according to Rudolph and Krueger (34) as modified from Laemmli (35). A separating gel (8.5% acrylamide) and a stacking gel (4% acrylamide) were used. Gels were stained with Coomassie Brilliant Blue R-250, destained in 45% methanol/10% acetic acid, and dried. Markers for molecular weight determination included phosphorylase B (M_r = 95,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 42,000), carbonic anhydrase (M_r = 30,000), and lysozyme (M_r = 14,000). Autoradiography was performed by exposing dried gels to Kodak XRP-5 medical X-ray film. Densitometry was performed on a Zeineh soft laser scanning densitometer. Peak areas were corrected by substracting background density of the individual lanes.

RESULTS

After incubation of soluble rat ovarian fractions in the presence of [γ-^{32}P]ATP and electrophoretic separation of the ^{32}P-labeled proteins, the autoradiograms shown in Fig. 1 were obtained.

Comparison of the phosphorylation patterns shows that the presence of Ca^{2+} at a free effective concentration of 2×10^{-4} M allowed greater ^{32}P incorporation into two proteins of M_r = 95,000 and 50,000 daltons (lane A) than was observed without Ca^{2+} (EGTA control, lane B). The inclusion of chlorpromazine, a phenothiazine derivative, together with Ca^{2+} (lane C) blocked the Ca^{2+}-dependent increase of ^{32}P incorporation (compare with lane A) without markedly altering the Ca^{2+}-independent phosphorylation pattern (compare with lane B).

A semiquantitative estimate of the relative increase of ^{32}P label into the M_r = 95,000 and 50,000 proteins in the presence of Ca^{2+} can be made after densitometry of the phosphorylation patterns (Fig. 2). Comparison of the peak areas shows an approximate 5.3-fold, and 2.3-fold increase respectively in the degree of phosphorylation of the 95,000 and 50,000 dalton proteins.

DISCUSSION

Our results demonstrate that several soluble rat ovarian proteins are phosphorylated by endogenous protein kinase. The phosphorylation of at least two of these proteins, proteins of 95,000 and 50,000 daltons, is modulated by Ca^{2+} thus establishing the existence of a Ca^{2+}-sensitive phosphorylation system in the rat ovary. It is interesting to note that trophic hormone stimulation of intact isolated bovine luteal cells results in phosphorylation of several proteins including a 95,000 dalton protein (20, 21) and a 50,000 dalton protein (21).

The precise enzymatic nature of the phosphorylation system remains to be established. In analogy to the established calmodulin control of Ca^{2+}-dependent protein kinase in other tissues (29-31, 33), it is conceivable that calmodulin also participates in the regulation of the ovarian Ca^{2+}-sensitive protein kinase system.

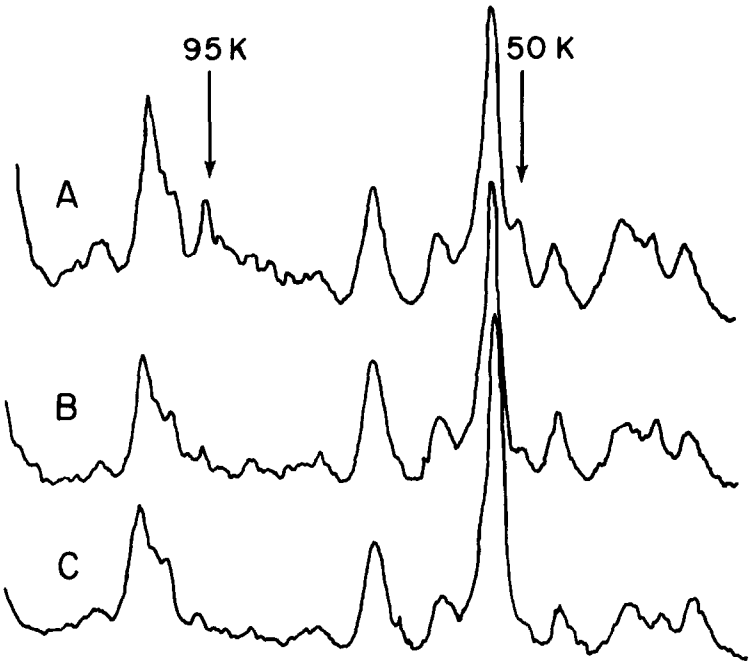

Figure 2. Densitometric tracings of autoradiograms shown in Fig. 1.

Inhibition of Ca^{2+}-dependent phosphorylation by chlorpromazine suggests calmodulin participation since phenothiazine derivatives can act as calmodulin inhibitors (36). Further studies into the role of calmodulin in regulation of Ca^{2+}-dependent phosphorylation in the rat ovary are being undertaken.

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