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INCREASED LEVELS OF A SPECIFIC CYCLIC-AMP BINDING PROTEIN IN ACTH STIMULATED BOVINE ADRENAL CORTICAL CELLS

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SUMMARY

Ion exchange chromatography of bovine adrenal cortex cytosol on DEAE-cellulose reveals two major cAMP binding protein fractions. Isolated adrenal cortical cell cytosol was found to contain these same two binding proteins although in different proportions. These two binding proteins can be distinguished by the inhibition of [^{3}H]-cAMP binding by a spectrum of cAMP analogues. The isolated adrenal cortical cells show an increase in total cAMP binding protein activity when stimulated with $ACTH_{1-24}$. This increase in total binding protein activity is due almost exclusively to one of the two binding proteins. Cycloheximide (10 μM) inhibits this ACTH stimulated cAMP binding protein.

INTRODUCTION

Although ACTH stimulated cAMP synthesis is correlated with steroidogenesis in the adrenal cortex, the molecular mechanism relating cAMP to steroid biosynthesis is unknown [1]. It is known that protein synthetic inhibitors such as cycloheximide block ACTH stimulated steroidogenesis without affecting ACTH stimulated increases in cAMP [2]. Studies such as these support the hypothesis that ACTH stimulates the synthesis of specific proteins which mediate steroidogenesis.

Increases in cAMP binding protein levels as a function of differentiation or transformation have been described in several systems [3–11]. In a mouse neuroblastoma cell line, differentiation is accompanied by a three-fold increase in soluble cAMP binding protein without a change in the cAMP dependent protein kinase levels [10, 11]. In view of these findings we have examined the possibility that a specific cytoplasmic cAMP binding protein is synthesized by adrenal cortical cells in response to ACTH stimulation.

Experimental

Preparation of adrenal cortex cytosol. Cortical tissue from fresh bovine adrenal glands was separated by dissection and homogenized in a medium consisting of 50 mM Tris–HCl, pH 7.4, 5 mM 2-mercaptoethanol, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ at 4°C and centrifuged at 9000 *g* for 15 min. The supernatant from this sedimentation was then centrifuged for 60 min at 105,000 *g*. The resultant supernatant was designated as cytosol.

Isolated adrenal cortical cells. Adrenal cortical cells were isolated from the digestion of decapsulated, demedullated bovine adrenal glands using 0.2% collagenase (Worthington) in Krebs–Ringer buffer containing 0.2% glucose and 0.5% bovine serum albumin at pH 7.4.

ACTH incubation. Before incubation with $ACTH_{1-24}$ (Cortrosyn, Organon, Inc.), the cell suspension was preincubated with the Krebs–Ringer buffer for 30 min at 37°C. $ACTH_{1-24}$ with or without 10 μM cycloheximide was then added to the incubation. At the end of the incubation the suspension was centrifuged and the supernatant used for cortisol determination. The cell pellet was then homogenized and centrifuged at 105,000 *g* for 60 min to obtain the cytosol fraction. The cytosol was dialyzed extensively before determining cAMP binding protein by the method of Prashad, *et al.*[10]. Samples were incubated with 0.25 μM [^{3}H]-cAMP (ICN, Irvine, CA, 13.7 Ci/mmol) for 16 h at 4°C. Protein bound and free [^{3}H]cAMP were separated by chromatography on Sephadex G-25 (PD-10, Pharmacia). AMP and adenine were tested for their ability to displace the binding of [^{3}H]-cAMP by inclusion of these inhibitors in the standard binding protein assay. Cortisol was determined by radioimmunoassay [12] using [^{3}H]-cortisol (NEN, Boston, Mass., 50 Ci/mmole); anti-cortisol antibody was purchased from Harbor General Hospital (Torrance, CA). Ion exchange chromatography on DEAE-cellulose (DE-52 Whatman Co.) was done using a 2 × 13 column equilibrated with 10 mM Tris–HCl, pH 7.4. Discontinuous NaCl gradients (0, 0.125 M, 0.25 M and 0.5 M) were used to elute the columns.

RESULTS

Figure 1 demonstrates that the isolated cells respond to ACTH by increasing cortisol synthesis

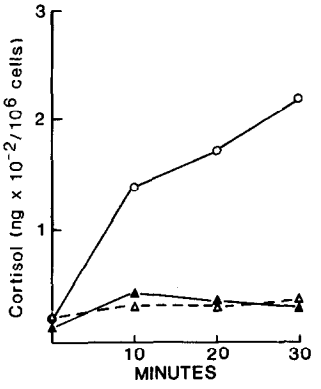

Fig. 1. The effect of ACTH and cycloheximide on cortisol synthesis in isolated cells. Control cells (▲----▲), or cells treated with 100 mU ACTH (○——○), or 10 μM cycloheximide + 100 mU ACTH (△----△), were incubated for various times and cortisol production measured.

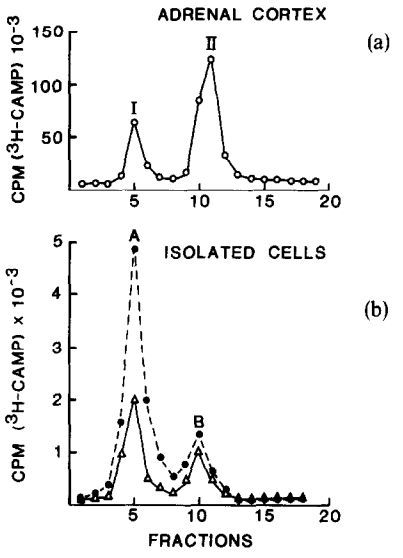

Fig. 2. The elution profile of cAMP binding proteins in DEAE-cellulose chromatography of cytosol from adrenal cortical tissue and isolated cells. Cytosol of adrenal cortex homogenate (a) and isolated cells (b) was chromatographed on DEAE-cellulose, and eluted with discontinuous NaCl gradients (0, 0.125 M, 0.25 M, 0.50 M). Aliquots of fractions were assayed for cyclic AMP binding protein. In (b), ●----●: 100 mU/ml ACTH; △——△: control cells.

approximately seven fold compared to basal cells at the end of a 30 min incubation. The maximum rate of cortisol synthesis is reached at least by 10 min. The addition of 10 μM cycloheximide at the same time as ACTH blocks ACTH stimulated steroidogenesis.

The effect of ACTH on cAMP binding protein was studied by incubating isolated cells with or without 100 mU $ACTH_{1-24}$ for 10 min. A 10 minute incubation period was chosen because cortisol synthesis had reached a maximum rate by that time. After incubation, the cells were washed and the extensively dialyzed cytosol was analyzed for binding protein as described in the experimental section. The results of seven such experiments are summarized in Table 1. cAMP binding protein was found to be increased in ACTH treated cells in each experiment. Increases ranged from 136% to 247% of control with a mean of 164%. Incubation of cells with 10 μM cycloheximide blocked this ACTH induced increase in binding protein in each experiment.

The total cAMP binding protein activity was analyzed by chromatography of isolated cell cytosol on DEAE-cellulose. Cytosol from adrenal cortex homogenate was also studied for comparison. Figure 2a shows that whole adrenal cortex cytosol has two major bands of cAMP binding protein, I and II, eluting at 0.125 M and 0.25 M NaCl, respectively. In Fig. 2b both control and ACTH stimulated isolated cells show two major peaks, A and B, eluting at the same positions as peaks I and II although in different proportions. It can be seen that ACTH stimulated cells show an increase in peak A compared to control cells while the amount of cAMP binding protein in peak B is approximately the same in ACTH treated and control cells. This ACTH stimulated increase in peak A with no change in peak B cAMP binding proteins was observed in every experiment, indicating that the increase in total cAMP binding protein observed in ACTH treated cells is due entirely to increases in peak A binding protein.

Further evidence for a functional difference between peak A and B binding proteins was sought

Table 1. Effects of ACTH and cycloheximide on cAMP binding protein levels in isolated cells

	% of Control				
	Cortisol	cAMP	cAMP BP*	(SEM)†	P‡
Control	100	100	100	—	—
ACTH	277	422	164	(16.1)	<0.01
ACTH and Cycloheximide	98	400	106	(1.0)	>0.5
Cycloheximide	81	96	81	(2.7)	<0.001

Isolated cells were incubated for 10 min at 37°C with 100 mU ACTH or 10 μM cycloheximide or both. Data is represented as per cent of control. The values are means from seven experiments done on seven different cell preparations.

* cAMP BP = cAMP binding protein.

† SEM = Standard error of the means.

‡ P values are calculated for cAMP binding protein difference from control.

Table 2. Displacement of [^{3}H]-cAMP binding to cAMP binding protein by unlabeled inhibitors

Inhibitor	Final concentration (μM)	% Inhibition I*	II*	ACTH†	Control†
3',5' cAMP	200	99	99	99	95
AMP	60	32	68	3	77
Adenine	120	2	44	2	56
2',3'-cAMP	30	45	70	4	15

* Peaks I and II from DEAE-cellulose chromatography of adrenal cortex cytosol—see Figure 2a.
† ACTH treated and control cell cytosol.

by a study of the inhibition of specific [^{3}H]-cAMP binding to these proteins by AMP, adenine and 2',3'-cAMP. Because of the extremely limited quantities of peaks A and B obtainable from isolated cell cytosol, the unfractionated cytosol from ACTH stimulated and control cells was used. These studies are shown in Table 2. Within the concentration ranges studied, the binding proteins from control cells were more sensitive to adenine and AMP (56% and 77% inhibition, respectively) than were the binding proteins from ACTH treated cells which showed no inhibition at the same concentrations of inhibitors. The results of similar inhibition studies done on peaks I and II from whole adrenal cortex cytosol are also included in Table 2. The inhibition spectrum shows that the binding protein from peak I and those from the cytosol of the ACTH stimulated cells are generally less sensitive to these inhibitors. This is evidence that the inhibition activities measured in the whole cytosol from ACTH treated cells represents predominantly peak A activity and the inhibition spectrum of control cytosol a mixture of A and B.

DISCUSSION

While cAMP is clearly an important regulator of ACTH stimulated steroidogenesis [2] and activation of adrenal protein kinases by this cyclic nucleotide has been correlated with corticosteroidogenesis [13], a correlation of cAMP levels with steroidogenesis or a mechanism for protein kinase activation of this process has not been established [2, 14, 15]. Apparently other factors either modulating the cAMP system or independent of it are also involved in regulation of corticosteroidogenesis.

We have shown that ACTH can increase the amount of a specific cAMP binding protein in isolated bovine adrenal cortical cells. Evidence for this conclusion is based on the following experimental results: (1) ACTH causes an increase in the total cytosol cAMP binding protein of isolated cells; (2) This increase is measurable during the same time period when ACTH stimulated cortisol synthesis is at a maximum rate; (3) The increase in ACTH stimulated cAMP binding protein is inhibited by cycloheximide, a known protein biosynthetic inhibitor, under these same conditions of cycloheximide inhibition, ACTH stimulated cortisol synthesis is blocked while the stimulated increase in cAMP is not; (4) Characterization of the cAMP binding proteins from basal and ACTH treated cells by DEAE-cellulose chromatography revealed that only one of the two major cAMP binding proteins is increased by ACTH stimulation; (5) Studies of inhibitor specificity for cAMP displacement indicate the existence of two distinct proteins.

It is possible that these observed increases in a particular cAMP binding protein may be involved in regulating ACTH induced steroidogenesis. Whether this particular cAMP binding protein operates by regulating a protein kinase or another regulatory system remains to be determined. It has been postulated that non-kinase associated cAMP binding proteins mediate cAMP effects by altering its intracellular concentration [16–21]. We have recently described the identification and partial characterization of a cAMP binding protein and cAMP dependent protein kinase from the plasma membranes of bovine adrenal cortex [22]. The present study did not determine whether changes in the membrane associated cAMP binding proteins occurred with ACTH stimulation but their existence offers an additional regulator of the effects of cAMP in these cells on steroidogenesis.

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Note added in proof: Preliminary studies [23] have previously shown an ACTH stimulated increase in [^{3}H]-cAMP binding in crude cytosol of Y-1 adrenal tumor cells.

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