ADENYL CYCLASE AND HORMONE ACTION

III. CALCIUM REQUIREMENT FOR ACTH STIMULATION OF ADENYL CYCLASE

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Summary: ACTH sensitivity of adenyl cyclase in mitochondrial and microsomal fractions from bovine adrenal cortex and in ghosts from rat fat cells can be abolished by the Ca complexing agent EGTA and restored by addition of Ca. EGTA also increases basal levels of cyclase activity in both systems and the degree of epinephrine stimulation in ghosts. Added Ca ion is inhibitory for basal and hormone stimulated activity in both systems. Ca may thus regulate 3',5'-AMP formation both in the absence and presence of hormones.

ACTH stimulates adenyl cyclase in membrane fractions derived from rat fat cells (1, 2), mouse adrenal tumor cells (3) and bovine adrenal cortex tissue (4). Adenyl cyclase in fat cell ghosts is stimulated by epinephrine and glucagon as well as by ACTH; the adrenal adenyl cyclase systems exhibit high selectivity to ACTH.

In previous work (2) it was found that Ca was required for the action of ACTH, but not of glucagon or epinephrine, upon fat cell ghost cyclase, as evidenced by the findings that the stimulatory effects of ACTH were selectively abolished by the Ca complexing agent, ethyleneglycol-bis (β-amino-ethylether)-N,N'-tetraacetic acid (EGTA), and restored by addition of Ca. In the present paper, results are presented which demonstrate that trace amounts of Ca are required for ACTH stimulation of adenyl cyclase in bovine adrenal membrane fractions as well as rat fat cell ghosts.

Methods and Materials: Fat cell ghosts were prepared according to the method of Rodbell (1) from rat epididymal fat pads, obtained from Sprague-Daley rats (140-200 g). Ghosts were suspended in 1 mM $KHCO_3$ containing 0.1% bovine serum albumin and stored at -70°. Purified mitochondria and a microsomal fraction (105,000 *g* pellet) from bovine adrenal cortex was prepared as

previously described (4). Adenyl cyclase assays contained in a total volume of 0.05 ml: 40 mM Tris-HCl, pH 8; 5 mM $MgCl_2$; 8 mM phosphoenol pyruvate (Sigma); 50-100 µg/ml pyruvate kinase (Sigma); 0.5 mM adenosine-3',5' cyclic monophosphate (3',5'-AMP, Sigma); 0.1% bovine serum albumin (Armour, Fraction V); 0.2-0.5 mg/ml protein of fat cell ghosts or adrenal fractions; and 0.1 mM ATP -α-^{32}P (International Chemical and Nuclear Corporation, about 1 x 10^6 cpm). Following incubation at 37° for 15-30 minutes, 0.05 ml of 5 mM ATP + 5 mM 3',5'-AMP was added and the tubes immersed in a boiling water bath for 3 minutes. Separation of products was performed on polyethyleneimin impregnated cellulose thinlayer sheets (Macherey-Nagel/Brinkman) using 0.3 M LiCl for development. The ATP and 3',5'-AMP spots were located by UV, cut out and measured by liquid scintillation counting. Details of this procedure have been recently published (5).

Synthetic ACTH (β^{1-24} adrenocorticotropic hormone, Synacthen) was a kind gift from Dr. W. Rittl, Basel, Switzerland and synthetic porcine glucagon from Dr. E. Jaeger, Max-Planck-Institut, München, Germany. L-epinephrine was purchased from Sigma.

Results: Table 1 shows that 1 mM EGTA inhibits the stimulatory effect of ACTH on adenyl cyclase in membrane fractions from bovine adrenal cortex; the sensitivity of adenyl cyclase to ACTH is restored upon addition of 1 mM $CaCl_2$ to the system containing 1 mM EGTA. In addition, EGTA increases basal cyclase activity, presumably by complexing Ca associated with the membrane system. Analogous observations have been documented with fat cell ghosts (2).

Adrenal cortex membrane fractions prepared in Ca-free media contain membrane bound Ca which apparently is released slowly from binding sites and then complexes with EGTA. Fat cell ghosts similarly possess membrane bound Ca. Ghosts prepared using a Ca-free medium for lysis of fat cells and ghosts prepared with the usual lysis medium which contains 0.1 mM Ca respond identically to EGTA in that ACTH sensitivity is lost between doses of 10^{-4} - 10^{-5} M EGTA.

An attempt was made to define the minimal concentration of Ca necessary for ACTH sensitivity of adenyl cyclase in fat cell ghosts and adrenal mito-

Table 1. EFFECT OF EGTA AND Ca ON ACTH SENSITIVITY OF BOVINE ADRENAL FRACTIONS

	pMoles 3',5'-AMP/min/mg protein					
			+EGTA		+EGTA +Ca	
Fraction	Basal	+ACTH	Basal	+ACTH	Basal	+ACTH
Mitochondria	17	41	27	28	20	43
	18	45	22	23	21	54
Microsomes	6.5	17.8	9.5	10.4	10.2	17.3
	5.4	19.2	13.6	10.2	10.7	18.5

Protein concentrations were 0.2 mg/ml with mitochondria and 0.32 mg/ml with microsomes. EGTA and $CaCl_2$ were each 1 mM where indicated, ACTH was 0.34 x 10^{-5} M. Values of duplicate determinations are listed.

chondria. When increasing amounts of $CaCl_2$ are added to assays containing 1 mM EGTA, pCa^{++} as a function of added $CaCl_2$ can be estimated using the acid dissociation and Ca complex constants of EGTA given by Chabarek and Martell (6), as shown in Figure 1-A. In repeated experiments with fat cell ghosts it was shown that about one equivalent of $CaCl_2$ (1 mM) must be added to restore ACTH sensitivity; a typical experiment is shown in Figure 1-B. Comparison of the curves for ACTH stimulated activity with the theoretical curve in Figure 1-A shows that ACTH sensitivity is detected when Ca concentration reaches about 10^{-8} - 10^{-7} M*. Results obtained with adrenal mitochondria (Figure 1-D) show that ACTH sensitivity is observed when the Ca ion concentration is 10^{-9} - 10^{-8} M*. Figure 1-C demonstrates that epinephrine and glucagon stimulation of cyclase in fat cell ghosts, in contrast to ACTH, is retained in the presence of

*The points in Figure 1-A have been calculated neglecting competition of Mg for EGTA as well as possible effects of temperature and ionic strength, i.e. the correlations made are estimates only.

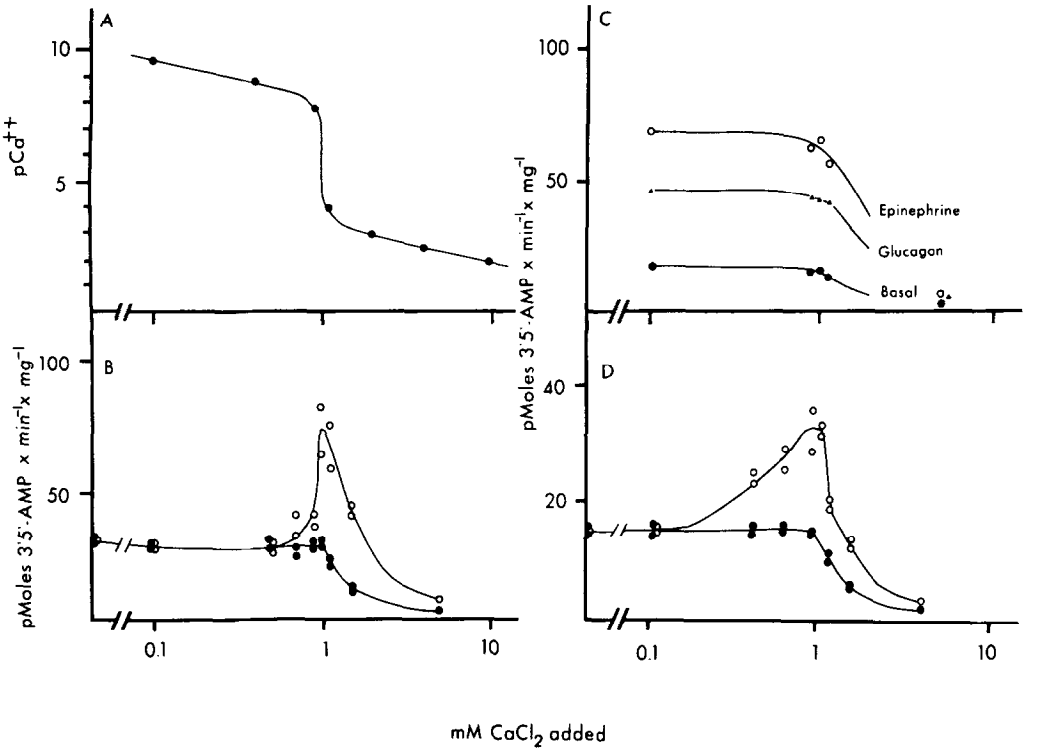

Figure 1. Ca Dependence of Hormone Sensitivity of Fat Cell Ghost and Adrenal Mitochondrial Cyclase A: Points below 1 mM $CaCl_2$ were calculated from data given by Chabarek and Martell (6), points above 1 mM $CaCl_2$ from the difference of mM $CaCl_2$ minus 1 mM. B: Ghost cyclase in the absence (closed circles) and presence of 0.3×10^{-5} M ACTH (open circles). C: Same as B, in the absence and presence of 10^{-4} M epinephrine and 0.3×10^{-5} M glucagon. D: Adrenal mitochondrial cyclase in the absence (closed circles) and presence of 0.3×10^{-5} M ACTH (open circles).

EGTA. A common finding was that addition of Ca above the equivalence point of 1 mM reduces basal and hormone stimulated cyclase activities in both fat cell ghosts and adrenal mitochondria (Figures 1-B, C, D). In other experiments addition of 0.1 - 2 mM $CaCl_2$ to both cyclase systems, assayed in the absence of EGTA, was found to reduce both basal activity and the degree of ACTH stimulation; similar inhibitory effects of Ca have been reported for membrane fractions obtained from mouse adrenal tumor cells (3).

In further studies, the effect of varying EGTA concentrations upon basal and hormone stimulated cyclase in fat cell ghosts was determined. Figure 2

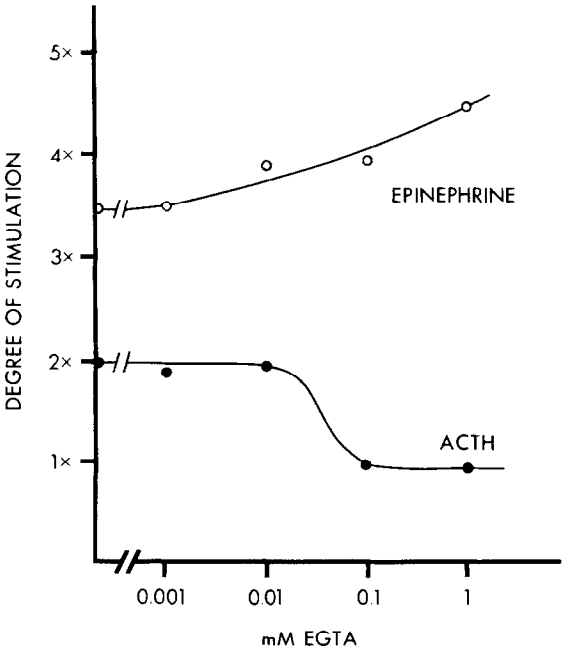

Figure 2. Hormone Sensitivity of Ghost Adenyl Cyclase as a Function of EGTA Concentrations Ghost cyclase was assayed at increasing doses of EGTA in the absence and presence of 10^{-4} M epinephrine and 0.3 x 10^{-5} M ACTH. On the abscissa, the ratio of hormone stimulated to basal cyclase activity is expressed.

shows that ACTH sensitivity is abolished at 0.1 mM EGTA. In contrast, the degree of epinephrine stimulation is increased by EGTA; the potentiation of epine phrine stimulation by EGTA has been consistently observed in additional experiments. Correlation of EGTA concentration with free Ca in these experiments could be made only if the total amount of membrane bound Ca as well as the capacity and binding constants of the membrane preparation were known.

Discussion: It is well established that Ca is required for the action of ACTH to promote steroidogenesis in adrenal sections (7) and lipolysis in rat fat pads (8). In both systems ACTH is believed to act by the generation of 3',5'-AMP (9). Mosinger and Vaughan suggested that Ca is required for an early event in ACTH action, since N^6,$O^{2'}$-dibutyryl 3',5'-AMP and theophylline both induced lipolysis in fat cells incubated in Ca-free medium. The present results which demonstrate that Ca at 10^{-9} to 10^{-7} M is required for action of

ACTH on adenyl cyclase, both in fat cell ghosts and in adrenal cortex membrane fractions, support this suggestion. Our experiments do not indicate whether trace amounts of Ca are required for the binding of ACTH to its selective membrane site (with "receptor" or "discriminator") or for a subsequent membrane event which leads to activation of adenyl cyclase.

Consideration of the effects of EGTA and Ca on adenyl cyclase in ghosts and adrenal cortex fractions suggests that Ca may play a general regulatory role in determining the rates of 3',5'-AMP formation in the absence and presence of hormones. The present findings do not support the idea that an ATP-Ca complex in the membrane serves as the substrate for adenyl cyclase (11).

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