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Adrenocorticotropin (ACTH) induces phosphorylation of a cytoplasmic protein in intact isolated adrenocortical cells

(hormone action/cyclic AMP/protein kinase/steroidogenesis)

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ABSTRACT **In ^{32}P incorporation experiments with intact adrenocortical cells, adrenocorticotropin (ACTH) or adenosine 3′,5′-cyclic monophosphate (cAMP) induced a rapid and transient increase of approximately 300–500% in the phosphorylation of a ^{32}P-containing cytoplasmic protein of about 150,000 daltons (APS_{150}). Half-maximal stimulation of APS_{150} phosphorylation was observed with about 3 pM ACTH. Receptor-bound cAMP, corticosterone production, and the appearance of phosphorylated APS_{150} increased in parallel with respect to both time and ACTH concentration. All three responses were dependent on extracellular calcium. Inhibition of protein synthesis with cycloheximide suggested a half-life of APS_{150} of about 10 min. The time course of ^{32}P incorporation into ACTH-induced APS_{150} in the absence and presence of nonradioactive phosphate shows that the phosphorylation of APS_{150} is under simultaneous control of cAMP-dependent protein kinase and of phosphatase activity. Thus a rapid ACTH-dependent and cAMP-dependent protein phosphorylation in intact adrenocortical cells within steroidogenic ACTH concentrations has now been demonstrated.**

It is well recognized that steroidogenesis in adrenal cortex and similar steroid-elaborating tissue is under complex hormonal control. In the current model of adrenocorticotropin (ACTH) action, it is proposed that the hormone binds to a specific receptor on plasma membrane and stimulates adenylate cyclase activity, with accumulation of adenosine 3′,5′-cyclic monophosphate (cAMP) and activation of a cAMP-dependent protein kinase (1). Until recently, the intermediate role of cAMP in the acute steroidogenic response to physiological concentrations of ACTH has been questioned (1). However, it has now been shown that hormonal stimulation of adrenocortical cells by ACTH in the steroidogenic concentration range is accompanied by a simultaneous increase in endogenous cAMP bound to the regulatory subunit of the protein kinase (2). Furthermore, studies with mutant clones of Y1 adrenocortical tumor cells (adenylate cyclase and cAMP-dependent protein kinase deficiency) provide additional evidence that cAMP and cAMP-dependent protein kinase are obligatory components of the ACTH-stimulated steroidogenesis pathway (3). In this context, the characterization of an endogenous specific substrate(s) for cAMP-dependent protein kinase(s) would substantially enlarge our understanding of the mechanism of acute ACTH action.

Several laboratories have reported phosphorylation of subcellular proteins in broken cells of adrenocortical tissue (4–8). Ribosomal protein phosphorylation stimulated by ACTH and cAMP in cultured mouse tumor cells was also described (9). However, this stimulation was achieved only by high ACTH concentrations. It has been shown that the activity of enzymes involved in steroidogenesis is increased by phosphorylation. Evidence has been presented that in adrenal cortex the phosphorylation of the cholesterol-ester hydrolase results in activation of cholesterol-ester hydrolysis, thus providing substrate cholesterol for the mitochondrial cholesterol side chain cleavage system (10, 11). However, it is now clear that this activation is not a rate-limiting process in steroidogenesis (11, 12). In ovary (13) but not in adrenal (14) a mitochondrial target site involving the cholesterol side chain cleavage system has been demonstrated. Nevertheless, in none of these studies has a physiological protein substrate(s) been shown to be phosphorylated in intact tissue in response to hormone. Because cAMP-dependent protein kinases do not appear to have a high substrate specificity (15, 16) and catalyze the phosphorylation of numerous substrates, such as histone, protamine, casein, phosphorylase kinase, lipoprotein lipase, glycogen synthetase, and RNA polymerase, and in addition these kinases autophosphorylate (17), it is important to achieve specificity by intracellular localization. This could be provided by the phosphorylation of a physiological protein substrate(s) in intact cells in response to physiological ACTH concentrations. This report demonstrates an ACTH-dependent and cAMP-dependent phosphorylation of an endogenous protein in intact isolated adrenocortical cells stimulated by ACTH in steroidogenic concentrations.

MATERIALS AND METHODS

Preparation and Incubation of Isolated Adrenocortical Cells of Zona Fasciculata-Reticularis Type. The digestion of decapsulated rat adrenals followed published procedures (18, 19). The cells were finally resuspended in Krebs–Ringer bicarbonate solution, phosphate-free, pH 7.4 (20). Incubations were done at 37°C as described (2) in triplicate or quadruplicate in 12 mm × 75 mm Falcon plastic tubes under O_2/CO_2 (19:1) with shaking (100 cycles per min) for different periods of time. The incubation was stopped by cooling the tubes in ice/water and all further processing was done at 0–2°C. Steroid assay, receptor-bound cAMP measurement, cAMP radioimmunoassay, and measurement of free and total cAMP receptor sites followed published procedures (2, 21, 22).

^{32}P-Labeled Phosphoprotein Formation. [^{32}P]Phosphate incorporation into adrenocortical protein was analyzed by methods similar to those previously described for adipocytes (23) with the modification described for hepatocytes (24) and adapted by us for adrenocortical cells. Cell suspensions (2×10^6 cells per ml; 3 mg of protein per ml) were equilibrated with [^{32}P]phosphate (400 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) for different times at 37°C or 0°C in a total volume of 0.7 ml; this was followed by addition of (final concentrations) 1-methyl-

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Abbreviations: ACTH, adrenocorticotropin (adrenocorticotropic hormone); cAMP, adenosine 3′,5′-cyclic monophosphate; Bt-cAMP, N^6-monobutyryl adenosine 3′,5′-cyclic monophosphate; APS_{150} ACTH-dependent phosphorylated protein; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

3-isobutylxanthine (0.3 mM), Synacthen [ACTH-(1–24); 0–180 pM; 1 fmol corresponds to 0.3×10^{-6} international units of corticotropin] or N^6-monobutyryl cyclic AMP (Bt-cAMP) (3.0 mM) or cAMP (10 mM). Cell suspensions and all solutions were made in phosphate-free Krebs–Ringer bicarbonate containing 0.01 M glucose and 0.5% (wt/vol) albumin. The final incubation volume was 0.9 ml. After incubation for 2–30 min, 2 ml of cold Krebs–Ringer bicarbonate solution, pH 7.4 containing 20 mM NaF, 10 mM EDTA, 150 mM potassium phosphate (homogenization buffer) was added and the ^{32}P-labeled cells were centrifuged at $500 \times g$ for 5 min. Cell pellets were washed once with 2 ml of homogenization buffer and finally resuspended in 1.0 ml of the same buffer. Cell suspensions were sonicated immediately at 100 W for 15 sec, and the sonicated extracts were precipitated with 2.0 ml of 10% trichloroacetic acid in water. The pellets were washed twice with 0.5 ml of 10% trichloroacetic acid, resuspended in 0.2 ml of electrophoresis sample buffer [3.3% sodium dodecyl sulfate ($NaDodSO_4$)/13.8% (vol/vol) 0.5 M Tris·HCl, pH 6.8/5.5% (vol/vol) mercaptoethanol/0.01% bromophenol blue/11.1% (vol/vol) glycerol], transferred to a boiling water bath for 2 min, and kept frozen until used. Samples (30 μl, 80 μg of protein) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis based on a published procedure (25) with minor modifications. The separating gel was a polyacrylamide slab gel (1 mm thick) containing a linear gradient of 5–17% acrylamide and *N,N'*-methylenebisacrylamide (30:0.8). Running buffer was Tris/glycine, pH 8.3/0.1% $NaDodSO_4$. The gels were prepared the day before use and electrophoresis was carried out at constant current (10 mA) and at initial 150 V for 6 hr. After electrophoresis the gels were immersed in 50% (wt/vol) trichloroacetic acid/water for 30 min, stained with 1% Coomassie blue in 50% trichloroacetic acid (30 min), and destained in acetic acid/methanol/water (1:10:89, vol/vol) overnight. The gels were dried and radioautograms were prepared and analyzed by densitometry. Quantitation of [^{32}P]phosphate incorporation (24) and statistical analyses (22) followed published procedures. The correlation between the amount of radioactivity in the gel and the densitogram was checked with a radioautogram of known increasing amounts of radioactivity. The incorporation of [^{32}P]phosphate into ACTH-dependent phosphorylated protein (APS_{150}) is expressed in arbitrary units. The values are integrated from the area quantitated by densitometry. They are expressed as a percent of the corresponding control value. To correct for intersample variation, the ratio of the peak height of three proteins (molecular weights 25,000, 70,000, and 100,000; heights were shown to be unaffected by the different incubation conditions used) from ACTH-treated cells to the peak height of the controls was used.

Sonication of cell suspensions in homogenization medium before or after trichloroacetic acid precipitation effectively inhibits ^{32}P turnover, as described for hepatocytes (24). Sonicated extracts of ^{32}P-labeled adrenocortical cells incubated at 4°C for 3 hr showed unchanged gel electrophoresis patterns of stained or ^{32}P-labeled protein in control and hormone-treated cells. This observation indicated that we could examine the pattern of ^{32}P-labeled protein after subcellular fractionation. In order to detect whether there was any ^{32}P incorporation during homogenization and preparation of the samples for electrophoresis, unlabeled cell pellets were resuspended in homogenization buffer containing [γ-^{32}P]ATP with specific activity similar to that achieved during [^{32}P]phosphate preincubations (assuming that practically all [^{32}P]phosphate is incorporated entirely into intracellular ATP). Preparation of samples for electrophoresis was as above. Six defined phosphorylated bands were observed, none of them corresponding to APS_{150}. There was no difference in the pattern of these ^{32}P-labeled proteins in cells incubated with or without ACTH and in cells sonicated in the presence or absence of 1 μM cAMP. Therefore, it was very unlikely that cAMP-dependent protein phosphorylation occurred after sonication. These phosphorylated protein bands observed in these experiments could be eliminated from gel autoradiograms by addition of nonradioactive ATP to the homogenization buffer.

The ^{32}P-labeled species obtained during adrenal cell incubations with or without hormone appear to consist exclusively of ^{32}P-proteins. The [^{32}P]phosphate was entirely released by immersion of the gels in 1 M NaOH, 90°C, for 10 min. Furthermore, incubation of sonicated extracts from ^{32}P-labeled cells with Pronase or trypsin (0.2 mg/ml, 15 min at 23°C) prior to electrophoresis resulted in gels devoid of ^{32}P after staining, whereas treatment with ribonuclease (0.2 mg/ml for 15 min at 23°C) had no effect on the ^{32}P pattern. Maximal [^{32}P]phosphate uptake by adrenocortical cells was achieved after 15 min at 37°C. The hormone-dependent formation of the ^{32}P-labeled protein APS_{150} was independent of the degree of [^{32}P]phosphate uptake by the cells.

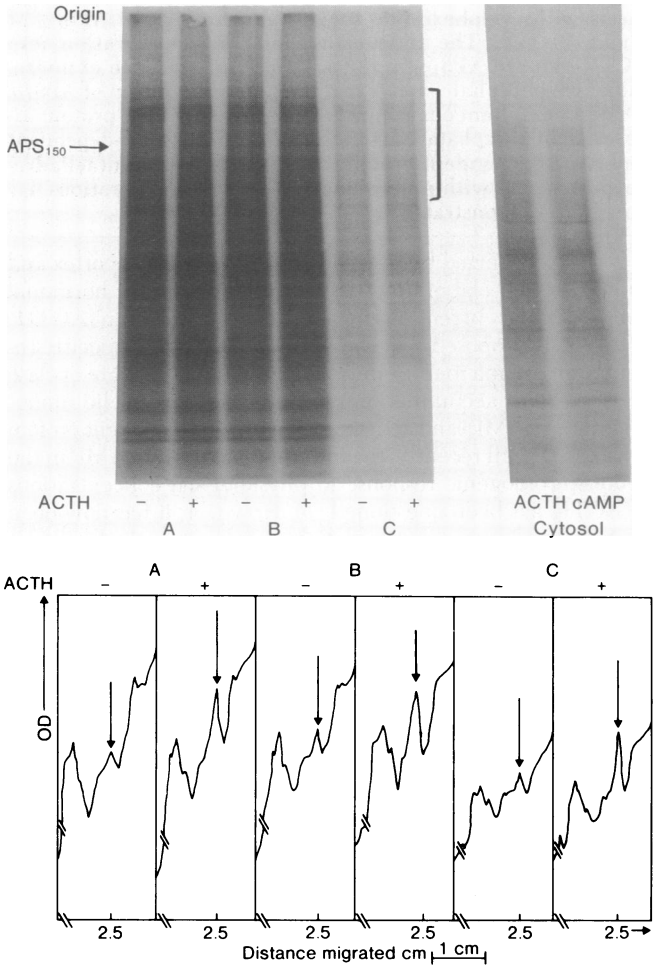

FIG. 1. (*Upper*) Autoradiogram of adrenocortical cell ^{32}P-protein after $NaDodSO_4$ slab gel electrophoresis. Cells were incubated with [^{32}P]phosphate for 30 min (A) or 15 min (B) at 37°C or for 30 min at 4°C (C). After preincubation, cells were incubated without (−) or with (+) 28 pM ACTH for 5 min at 37°C. The bracket indicates the zone of the autoradiograms illustrated in the densitograms of *Lower*. The autoradiograms on the right represent adrenocortical cytosol from cells incubated with 28 pM ACTH or 10 mM cAMP. (*Lower*) Densitograms of autoradiogram from *Upper*.

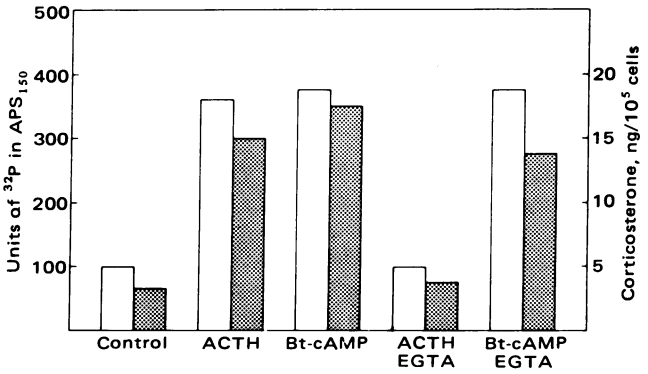

FIG. 2. Concomitant phosphorylation of APS_{150} (open bars) and corticosterone production (stippled bars) under various conditions of stimulation. [^{32}P]Phosphate preincubation for 30 min at 4°C; hormone incubation 15 min. APS_{150} is expressed in arbitrary ^{32}P units. EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

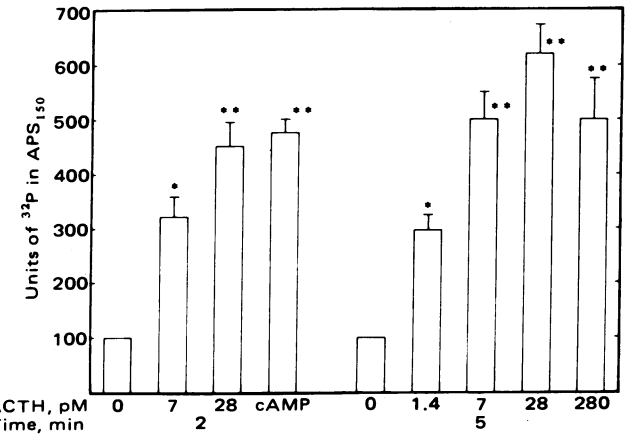

FIG. 3. Time course and dose response of hormone-stimulated APS_{150} phosphorylation. Experimental conditions are as in Fig. 2. The values from hormone-treated cells were analyzed statistically by Student's *t* test for paired data versus control. Values are means ± SD, $n = 4$. *, $P < 0.05$; **, $P < 0.01$.

RESULTS

[^{32}P]Phosphate Incorporation into Intact Adrenocortical Cells. ^{32}P-labeled proteins from cells incubated in the absence and the presence of ACTH were analyzed by polyacrylamide/$NaDodSO_4$ gel electrophoresis. The staining patterns were identical and the autoradiograms were also identical except for an increase in phosphorylation of a protein in the case of ACTH-stimulated cells. This protein had an apparent molecular weight of about 150,000 and was designated APS_{150} (ACTH-dependent phosphorylated protein). As shown in Fig. 1 *upper*, ACTH brought about a rapid stimulation of APS_{150} phosphorylation by approximately 300–500%, which was independent of the relative [^{32}P]phosphate uptake by the cells. When adrenocortical cells (treated with 28 pM ACTH or 10 mM cAMP) were disrupted and homogenized in homogenization buffer (see *Materials and Methods*), subcellular fractionation of ^{32}P-labeled adrenocortical cells showed APS_{150} only in the 105,000 × *g* supernatant. Fig. 1 *lower* shows the quantitation of APS_{150} by densitometry. APS_{150} was phosphorylated to a much lesser degree in the absence of ACTH. The amount of ^{32}P incorporated into APS_{150} correlated well with the amounts of corticosterone and receptor-bound cyclic AMP found in both unstimulated and stimulated cells. Although with the exception of APS_{150} no significant changes were found in the densitograms of gels from control and ACTH-treated cells, we cannot rule out the possiblity that ACTH increases the phosphorylation of more than one protein.

Calcium-Dependent Increase in Phosphorylation of APS_{150} and Corticosterone Production. Fig. 2 shows APS_{150} phosphorylation and corticosterone production in adrenocortical cells stimulated by ACTH (28 pM). At physiological ACTH concentrations, steroidogenesis is known to be dependent on extracellular calcium, which was also found to be necessary for the phosphorylation of APS_{150} by ACTH. This is in contrast to Bt-cAMP, which stimulated both steroidogenesis and APS_{150} phosphorylation even in the presence of EGTA. Verapamil, an inhibitor of membranal calcium transport, brings about an equivalent inhibition of steroid production and APS_{150} phosphorylation (data not shown).

Changes in Phosphorylation of APS_{150}, Amount of Receptor-Bound Cyclic AMP, and Production of Corticosterone during ACTH Stimulation. cAMP bound to the regulatory subunit of the protein kinase is an excellent measure for the activity of cAMP-dependent protein kinase in intact cells (2, 21, 22). If APS_{150} had a mediating role in the steroidogenic action of ACTH, its phosphorylation should parallel receptor-bound cAMP and corticosterone temporally, and also with respect to ACTH threshold concentrations. Fig. 3 shows that ACTH (7 pM) induced APS_{150} phosphorylation within 2 min and the maximal level was reached within 5 min, persisting unchanged throughout 15 min (data not shown). APS_{150} phosphorylation was significantly stimulated with ACTH as low as 1.4 pM (5 min), with a half-maximal stimulation of about 3 pM. In response to ACTH, both the amount of receptor-bound cAMP and production of corticosterone (Table 1) correlated well with APS_{150} phosphorylation temporally and with respect to ACTH concentration (Fig. 3). These results strongly argue that APS_{150} is part of the physiological ACTH steroidogenesis pathway.

Time Course of APS_{150} Phosphorylation and Dephosphorylation. Maximal stimulation of APS_{150} phosphorylation was achieved within 5–15 min with a return to control values within 30 min (Fig. 4). Addition of nonradioactive phosphate, 5 min after ACTH stimulation, produced a marked decrease in the ^{32}P content in APS_{150}. This decrease was not specific for APS_{150} and was observed for all ^{32}P-protein in the gel (data not shown).

Table 1. Assay of bound cAMP and corticosterone in adrenocortical cells stimulated by increasing concentrations of ACTH

ACTH, pM	Bound cAMP, fmol/10^5 cells			Corticosterone, ng/10^5 cells		
	2 min	5 min	10 min	2 min	5 min	10 min
0	50.6 ± 3.5	50.6 ± 3.0	52.7 ± 3.0	1.9 ± 1.0	3.2 ± 1.0	7.8 ± 2.9
0.27	50.7 ± 4.7	50.3 ± 4.6	69.3 ± 3.6*	2.3 ± 1.5	5.4 ± 3.0	19.1 ± 2.3**
2.7	59.5 ± 3.0*	65.6 ± 5.2*	87.5 ± 6.0**	2.5 ± 3.4	8.6 ± 3.4**	28.6 ± 5.0***
27	103.1 ± 7.2***	152.5 ± 29.0***	134.2 ± 9.9***	3.2 ± 1.0	10.9 ± 3.4***	31.7 ± 2.9***

Values are mean ± SD; $n = 4$. In comparison with 0 pM ACTH, *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

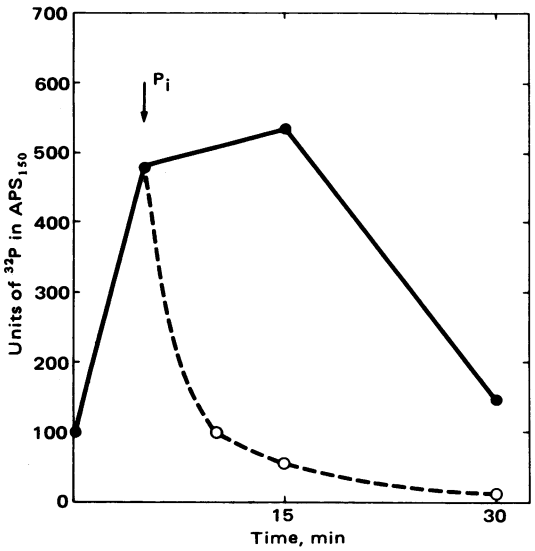

FIG. 4. Time course of APS_{150} phosphorylation and dephosphorylation. Cells were preincubated with [^{32}P]phosphate for 30 min at 4°C and then incubated without or with 28 pM ACTH (for 30 min). Hormone-treated and non-hormone-treated cells were chased with nonradioactive phosphate (0.150 M) 5 min after hormone stimulation. Units of ^{32}P in APS_{150} in absence (●) or presence (○) of nonradioactive phosphate (chase experiment). Values are means of close duplicates from one of two similar experiments.

Inhibition of Protein Synthesis in ACTH-Stimulated Cells. In adrenocortical cells incubated with cycloheximide (10 μM) prior to the hormonal stimulus (Table 2), the synthesis of corticosterone was completely inhibited after 5 min preincubation with cycloheximide; phosphorylation of APS_{150} was inhibited by only about 17%. The half-life of APS_{150} was about 10 min. The parameters of protein kinase activation (bound cAMP and free cAMP receptor sites) were not affected by cycloheximide treatment.

DISCUSSION

These studies have clearly demonstrated that stimulation of intact isolated adrenocortical cells by ACTH at concentrations in the steroidogenic range is accompanied by an ACTH-dependent cAMP-dependent phosphorylation of an endogenous protein. The results indicated that this phosphoprotein, APS_{150}, may be an important intermediate in the mechanism of ACTH action.

The hormone-induced phosphorylation of the cytoplasmic protein APS_{150} is rapid in onset and transient. These features argue strongly that the course of [^{32}P]phosphate incorporation reflects the simultaneous or sequential activity of a protein kinase and a phosphatase, rather than alternative mechanisms such as changes in [^{32}P]phosphate entry, specific activity of [γ-^{32}P]ATP, or protein synthesis and degradation. If APS_{150} phosphorylation were due to an ACTH-dependent change in the specific activity of the ATP donor pool, a selective change should be observed in the case of the chase experiments. [^{32}P]Phosphate incorporation into APS_{150} was not selectively inhibited by reduction of the specific activity of [^{32}P]phosphate by addition of nonradioactive phosphate. This strongly argues that the increased phosphorylation of APS_{150} is not due to changes in specific activity of the donor pool.

Recent evidence strongly demonstrates that cAMP and cAMP-dependent protein kinase are obligatory components of ACTH-stimulated steroidogenesis (2, 3). APS_{150} phosphorylation as a physiological intermediate has to fulfil several criteria: (*i*) ACTH should be capable of increasing APS_{150} phosphorylation in intact cells; (*ii*) the threshold dose of ACTH for cAMP increases, steroidogenesis and APS_{150} phosphorylation should be the same; (*iii*) the increase in the phosphorylation of APS_{150} should precede and not follow the physiological response to ACTH; (*iv*) because extracellular calcium is required for ACTH-induced activation of adenylate cyclase, which leads to an increase of intracellular cAMP and receptor-bound cAMP (unpublished data), the stimulation by ACTH of APS_{150} phosphorylation should be calcium dependent, and the stimulation by exogenous cAMP of APS_{150} phosphorylation should be calcium independent. ACTH stimulation of APS_{150} phosphorylation indeed fulfilled the criteria mentioned above. It is a rapid process (observable within 2 min) with a half-maximal stimulation at about 3 pM ACTH. Half-maximal stimulation of production of both receptor-bound cAMP and corticosterone is observed at about 1 pM (2). This small difference was due to a decrease in sensitivity of the system towards ACTH when phosphate was reduced in the incubation medium (data not shown). ACTH stimulation of APS_{150} phosphorylation is dependent on extracellular calcium, whereas cAMP stimulation of APS_{150} phosphorylation is not.

The cAMP receptor protein related to the steroidogenic response appears to be confined to the cytoplasm (21). The alteration of a cytosolic cAMP-dependent protein kinase activity in a mutant Y1 adrenocortical tumor cell line follows the alteration in the steroidogenic response to ACTH (3). These observations imply that the activation of protein kinase during hormone action is mainly a cytoplasmic event. The finding that APS_{150} is confined to the cytosol fraction suggested that both ACTH-dependent activation of protein kinase and phosphorylation are restricted to the cytoplasmic compartment. This localization is in contrast to the hypothesis proposing the mitochondrion as a target for phosphorylation (7), but it is in agreement with a previous suggestion that acute stimulation by ACTH of corticoid production does not involve mitochondrial phosphorylation (14). It is unlikely that APS_{150} represents an autophosphorylated kinase as described in other tissue (17) because the molecular weight is clearly different.

The inhibitory effects of cycloheximide on the activation of adrenal steroidogenesis have been known for many years (26, 27), and the rapid kinetics of inhibition have led to the hy-

Table 2. Effect of cycloheximide on ACTH stimulation of adrenocortical cells

Preincubation time, min	% of control (unstimulated cells)			
	Corticosterone	Bound cAMP	Free cAMP receptor sites	Units of ^{32}P in APS_{150}
0	875 ± 17	355 ± 39	55.8 ± 8.0	430 ± 33
5	103 ± 5	400 ± 81	62.1 ± 2.6	360 ± 18
15	105 ± 3	547 ± 39	66.9 ± 7.1	203 ± 15
30	103 ± 2	601 ± 44	64.2 ± 1.0	102 ± 3

Cells were preincubated with cycloheximide (10 μM) for 0–30 min and then incubated 15 min with 28 pM ACTH. Values = (+ACTH/−ACTH) × 100 (mean ± SD; n = 3).

pothesis that a labile protein (half-life about 2–4 min) is required for ACTH action (28, 29). Although there is good evidence for a role of protein synthesis in the mechanism of action of ACTH, a translational control by ACTH of protein synthesis *de novo* appears most unlikely in the case of acute stimulation (28, 29). It was suggested that ACTH indirectly transforms an inactive precursor protein to the active labile one (28) by phosphorylation (29). The turnover of phosphorylated APS_{150} appeared to be relatively fast (10 min half-life) but slower than that of the labile protein mentioned above. This may imply that the labile protein and APS_{150} are two different entities. This does not rule out the possibility that the labile protein can also be phosphorylated. In addition, it should be pointed out that, because cycloheximide inhibits protein synthesis, we cannot rule out the possibility that we have a rapid phosphatase turnover which could lead to a longer half-life of phosphorylated APS_{150}.

It is assumed from Fig. 4 that stimulation of APS_{150} phosphorylation is dependent on protein kinase and phosphatase activation. The decrease observed in APS_{150} after 15-min incubation with ACTH cannot be explained by a decrease in the [^{32}P]phosphate donor or protein substrate pools, because preincubation for 30 min before hormone addition still allows the detection of APS_{150} phosphorylation upon hormone treatment (see Fig. 1 *upper*, A). cAMP-dependent protein kinase was not shut off and its activation persisted for 30 min, as shown in Table 2 by the receptor-bound cAMP values and in previous reports in adrenal (2, 30, 31) and other steroid-elaborating tissue (32, 33). The marked decrease in ^{32}P incorporation in the case of the chase experiment (see Fig. 4) suggests that kinase and phosphatase act simultaneously or sequentially. The balance between the two enzymes is reflected in the amount of the phosphorylated (0- to 15-min hormone treatment) or dephosphorylated (15- to 30-min hormone treatment) forms of the endogenous substrate. This interpretation implies an additional consideration—i.e., a putative regulation of phosphatase activity by ACTH. It is clear that knowledge of the extent of protein kinase activity is not enough to predict the extent of protein phosphorylation. The further characterization of APS_{150} and the detection of the corresponding dephosphorylated substrate may substantially enlarge our understanding of ACTH action.

Note Added in Proof. The stimulation of phosphorylation of an additional protein (about 16,000 daltons) was observed when incubations were carried out with at least 280 pM ACTH or 3 mM Bt-cAMP for more than 15 min.

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