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ACTIVATION OF MICROSOMAL *c*AMP-DEPENDENT PROTEIN KINASE ISOENZYME I BY $ACTH_{1-24}$ IN BOVINE ADRENAL CELLS

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Summary—In microsomes of bovine fasciculata reticularis cells incubated with or without 10^{-8} M ACTH during 20 min, we measured covalent and non covalent *c*AMP binding under exchange or non-exchange conditions and *c*AMP-kinase activity. ACTH induced a decrease in *c*AMP-kinase activity and in the number of free *c*AMP binding sites. These results indicate an activation by ACTH of a part of microsomal *c*AMP-dependent protein kinase. Photoaffinity labeling of microsomal protein with 8-azido-*c*AMP revealed the presence of both *c*AMP-kinase isoenzyme I and II in this cellular fraction. Using this method, it was demonstrated that $ACTH_{1-24}$ caused a preferential and nearly complete activation of microsomal protein kinase I.

INTRODUCTION

Recently, we observed [1] a modification of *c*AMP-kinase subcellular distribution induced by $ACTH_{1-24}$ in bovine fasciculata reticularis cells. The particulate *c*AMP-kinase was decreased with only minor changes in particulate *c*AMP binding. In addition, as was already observed in other cell types [2–5], we found a compartmentalization of *c*AMP-kinase isoenzymes in fasciculata cells [6]. Isoenzyme I, but not isoenzyme II was found mainly in the cytosol whereas both isoenzymes were found in the microsomal fraction. Since both isoenzymes are present in microsomes, the question arises whether ACTH stimulation induces activation of one or both isoenzymes. In this study we investigated the covalent and the non-covalent binding of *c*AMP to microsomes derived from control and ACTH stimulated fasciculata reticularis cells.

EXPERIMENTAL

Adrenal fasciculata-reticularis cells were prepared from fresh bovine adrenal glands as described previously [1]. Cells (2×10^6 cells/ml) were preincubated for 2 h at 37°C in MEM supplemented with 20 mM Hepes pH 7.3, 0.25% BSA, 0.5 mM IBMX. Cells were incubated at 37°C for 20 min with agitation, with or without 10^{-8} M $ACTH_{1-24}$ (Synacthen–Ciba Geigy, Basel). Cells were pelleted, resuspended in 10 mM phosphate buffer, pH 6.8, containing 250 mM sucrose, 6 mM 2-mercaptoethanol, 0.5 mM IBMX and 250 mM NaCl, then homogenized in a Potter–Elvehjem homogenizer (25 strokes). The microsomal fraction was obtained as described elsewhere [6].

*c*AMP-Kinase activity and non-covalent *c*AMP binding were determined as described previously [1]. *c*AMP binding was measured by incubating microsomal proteins for 1 h at either 4 or 32°C with 4×10^{-8} M [^{3}H]*c*AMP (36 Ci/mmol, from Amersham, England).

Photoaffinity labeling of the microsomal fraction was done with 8-azido [^{32}P]*c*AMP (70 Ci/mmol, from I.C.N., U.S.A.) as described in detail previously [6]. Briefly, microsomes (20 μg of protein) were incubated with 8-azido [^{32}P]*c*AMP (2 to 5×10^{-7} M) with or without 10^{-5} M *c*AMP in 50 μl of 50 mM MES pH 6.2, 10 mM $MgCl_2$ and 1 mM IBMX. Preincubation was carried out in the dark for 1 h at either 4 or 32°C, followed by u.v. irradiation for 15 min at 4°C. Treatment of sample, polyacrylamide gel electrophoresis, and autoradiography were done as described previously [6].

RESULTS

Incubation of adrenal cells with 10^{-8} M ACTH resulted in a 40% decrease in microsomal *c*AMP-kinase activity (Table 1). *c*AMP binding measured at 4°C was also reduced to about the same extent. However, when this determination was performed at 32°C, the binding was only 17% lower in ACTH treated microsomes than in control. In all experimental conditions, preincubation of microsomes with

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Abbreviations: $ACTH_{1-24}$: Corticotropin (1–24) tetra- cosapeptide; *c*AMP: adenosine-3′,5′-cyclic monophosphate; *c*AMP-kinase: adenosine 3′,5′-monophosphate-dependent protein kinase; 8-azido-*c*AMP: 8-azidoadenosine 3′,5′-monophosphate; IBMX: isobutylmethylxanthine.

Table 1. cAMP-kinase activity and cAMP binding in microsomal fraction from control and $ACTH_{1-24}$ treated cells

	cAMP-kinase activity (pmol ^{32}P/min/mg protein)	[^{3}H]cAMP binding (pmol/mg protein)			
		No detergent		Triton X-100	
		+4°C	+32°C	+4°C	+32°C
Control	941 ± 97	6.1 ± 0.60	6.4 ± 0.13	7.7 ± 0.65	7.4 ± 0.30
$ACTH_{1-24}$	584 ± 143*	3.3 ± 0.40*	5.3 ± 0.80	6.0 ± 0.80*	6.7 ± 0.25*
$\frac{ACTH_{1-24}}{Control}$	0.63	0.55	0.83	0.77	0.90

Bovine adrenal cells were incubated with or without 10^{-8} M $ACTH_{1-24}$ for 20 min at 37°C and microsomal fractions were prepared. cAMP-kinase activity was measured in the presence of 10^{-6} M cAMP. Binding was determined after a 30 min preincubation with or without 0.25% Triton X-100. Results are means ± SEM of 3 different experiments. Asterisks indicate a significant decrease relative to control (Student's t-test: $P < 0.05$).

Table 2. Ratios of 8-azido-[^{32}P]cAMP incorporation in microsomal isoenzyme I and isoenzyme II bands in $ACTH_{1-24}$ stimulated cells relative to control cells

	Isoenzyme I (48,000)		Isoenzyme II (55,000)	
Temperature of photoaffinity labeling	Densitometry	Gel counting	Densitometry	Gel counting
+4°C	0.19 ± 0.05*	0.27 ± 0.11*	1.16 ± 0.23	0.91 ± 0.07
+32°C	0.76 ± 0.08*	1.075 ± 0.25	1.28 ± 0.05*	1.08 ± 0.22

Bovine adrenal fasciculata-reticularis cells were incubated with or without 10^{-8} M $ACTH_{1-24}$. Microsomal fractions were prepared, photoaffinity labeled at +4°C or +32°C and submitted to electrophoresis. Specific [^{32}P]cAMP incorporation in 48,000 and 55,000 Dalton bands was determined by autoradiogram densitometry and by scintillation counting of the bands. Numbers represent: incorporation in $ACTH_{1-24}$ cells/incorporation in control cells and are means ± SEM of 3 different experiments. Asterisks indicate that ratio is significantly different from 1 (Student's t-test $P < 0.05$).

Triton X-100 increased the binding of cAMP. However, the more marked increase was observed in microsomes assayed at +4°C, from ACTH treated cells. These results showed that addition of ACTH resulted in activation of microsomal cAMP-kinase. This activation was shown by a decrease of free cAMP receptor sites. It was also obvious that not every microsomal cAMP-kinase became activated following ACTH stimulation.

In order to determine whether both microsomal enzymes were activated by ACTH, microsomes were labeled covalently by photoaffinity with 8-azido-cAMP under the same conditions as for non-covalent binding. In a previous work [6] we identified clearly two main labeled components of 48,000 Daltons and 55,000 Daltons corresponding respectively to isoenzyme I and isoenzyme II regulatory subunits. When compared to control, isoenzyme I labeling (48,000 Dalton band) from ACTH treated microsomes was reduced by 80% if incubation was performed at 4°C (Fig. 1 and Table 2). However, at 32°C the reduction of isoenzyme I labeling was

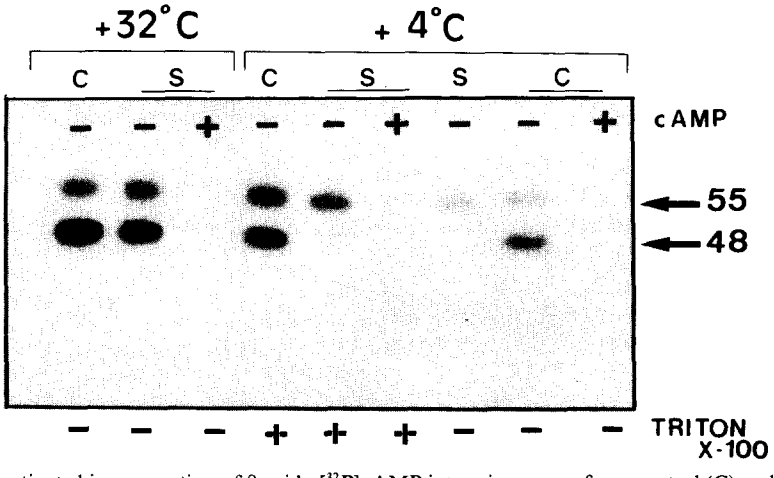

Fig. 1. Photoactivated incorporation of 8-azido [^{32}P]cAMP into microsomes from control (C) and ACTH treated cells (S). Experimental conditions were the same as in Table 2. In addition results of photoaffinity labeling after Triton X-100 preincubation (30 min at +4°C under agitation, 0.25% final concentration) were shown.

lower, reaching at most 24%. For isoenzyme II (55,000 Dalton band) no important difference was observed between control and hormone stimulated cells when photoaffinity labeling was performed either at 4°C or at 32°C. Pretreatment of control microsomes with 0.25% Triton X-100 enhanced the labeling of isoenzyme I (30%) and of isoenzyme II (180%) at 4°C (Fig. 1) and 32°C (data not shown). By contrast, the same pretreatment of microsomes from ACTH stimulated cells produced a reduced stimulation of isoenzyme II labeling at 4°C (120% versus 180% for control microsomes) and an absence of stimulation at 32°C. Even more, treatment with Triton X-100 reduced the labeling of microsomal isoenzyme I from ACTH stimulated cells.

DISCUSSION

This work confirms and extends our previous findings [1]: in bovine adrenal cells, ACTH causes a decrease in particulate cAMP kinase (by 40% in the experimental conditions reported here). A parallel diminution of cAMP binding is not observed when binding was determined at 32°C, a temperature which promotes exchange of added [^{3}H]cAMP with the endogenous cAMP bound to regulatory subunits [9]. However, when measured at 4°C to reduce such exchange, the cAMP binding of microsomes is decreased by ACTH (Table 1). This diminution in free cAMP binding sites indicates that ACTH activates a part of microsomal cAMP-kinase. As a consequence, the translocation of free catalytic subunits from microsomes to another cellular compartment occurs very likely.

Photoaffinity labeling with 8-azido-cAMP confirms that free cAMP binding sites are diminished in microsomes from ACTH cells (Table 2). In addition, selective reduction of microsomal isoenzyme I labeling measured at 4°C is demonstrated. (Table 2 and Fig. 1). Thus, microsomal isoenzyme I is nearly totally activated in cells incubated with 10^{-8} M ACTH for 20 min. However, these results do not preclude any activation of microsomal isoenzyme II during ACTH stimulation. Activation of isoenzyme II may occurs very early and for a limited period of time after ACTH stimulation [7]. Also, despite the presence of 0.25 M NaCl in the homogenization medium, partial reassociation of protein kinase II during the preparation of microsomes cannot be completely excluded [8]. The different effects of Triton X-100 on microsomes from control and ACTH cells remain presently unexplained.

The selective activation of cAMP-kinase isoenzyme I has been reported for several other hormonally responsive cells systems including rat Leydig cells/hCG [9], rabbit ovarian corpora lutea/hCG [10], mouse pituitary tumor cells/CRF [11] and rat liver/glucagon [7]. In rat adrenal, Koroscil *et al.*[12] observed a decrease in 8-azido-cAMP binding to microsomal isoenzyme I after injection of ACTH. To explain this effect they suggest two possibilities, either an increase of endogenous cAMP or a translocation of isoenzyme I regulatory subunit. Our results of photoaffinity labeling at 2 temperatures indicate clearly that in bovine adrenal cells regulatory subunit translocation was not responsible for the decrease in microsomal isoenzyme I labeling. In fact, our data demonstrate that ACTH stimulation produced an activation of microsomal isoenzyme I. Specific implication of this isoenzyme in ACTH stimulated adrenal steroidogenesis was also documented by Doherty *et al.*[13]. They found in some mutants of Y1 cells, resistant to ACTH a modification affecting specifically the isoenzyme I regulatory subunit. All these results prompt further studies to characterize the structure (i.e. rough endoplasmic reticulum, Golgi apparatus, cytoskeleton) bearing each isoenzyme in the heterogenous microsomal fraction. Moreover, modifications in microsomal protein phosphorylation should be studied, in order to better understand the sequence of events taking place between the initial ACTH induced cAMP rise and the final increase in pregnenolone synthesis.

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