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THE ROLE OF PROTEIN KINASES IN ACTH-STIMULATED STEROIDOGENESIS

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The effect of highly purified bovine cytosolic adrenal cortical protein kinase isozyme II catalytic subunit (ATP: Protein Phosphotransferase EC 2.7.1.37) on the formation of pregnenolone from cholesterol in rat and bovine adrenal mitochondrial extracts has been investigated. No stimulation was observed although a low level incorporation of [^{32}P] from [^{32}P]-ATP into a component or components of the extract was detected. The mitochondrial extracts contained alkaline phosphatase activity that was inhibited by L-cysteine and dithiothreitol. It is concluded that the acute stimulation by ACTH of corticoid production in the rat adrenal does not involve protein kinase mediated phosphorylation of a component or components of the cholesterol sidechain cleavage mixed-function oxygenase system.

Several hypotheses, none entirely satisfactory, have been advanced to explain the acute action of ACTH on the adrenal cortex i.e. the stimulation of corticoid formation from cholesterol (desmolase reaction). The initial, rather well substantiated, steps in ACTH action in the adrenal cortex are binding of the hormone to receptors on the surface of the plasma membrane and activation of adenylate cyclase to form 3',5'-cyclic AMP (c-AMP) (1-3). The cyclic nucleotide in turn appears to activate a protein kinase or protein kinases (4). The conversion of cholesterol to 5-pregene-3β-ol-17-one (pregnenolone) takes place in the mitochondrion and is catalyzed by a cytochrome P_{450} type mixed function oxygenase system (5). It is this step

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Abbreviations: ACTH=adrenocorticotropic hormone; BSA=bovine serum albumin; DTT=DL-dithiothreitol; TCA=trichloroacetic acid; U=unit(s).

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in the sequence of reactions leading to the formation of corticoids that is under ACTH control (6). In the chain of reactions through which ACTH regulates corticoid production in the adrenal cortex the link between the initial events i.e. activation of adenylate cyclase and protein kinase(s) and the final phase i.e. the regulation of the cholesterol desmolase system remains to be discovered.

In the present investigation we explore the effect of highly purified bovine cytosolic protein kinase isozyme II catalytic subunit on the rat mitochondrial cholesterol sidechain cleavage system.

Materials. Bovine protein kinase isozyme II catalytic subunit was prepared by the method of Finn et al. (to be published). 4-[^{14}C]-cholesterol was from New England Nuclear Corporation. Hepes, D-glucose-6P, NADP, glucose-6P-dehydrogenase, cholesterol, pregnenolone, progesterone, DTT, histone IIA, and p-nitrophenyl phosphate were from Sigma. Silica gel G (type 60) was from E.M. Laboratories. Trilostane was a gift from the Sterling-Winthrop Research Institute. Solvents were of analytical grade.

Methods. Mitochondria were prepared from adrenals of male Sprague-Dawley rats weighing approximately 300 g by the method of Simpson et al. (7). Acetone powders were prepared according to Halkerston et al. (8). For preparation of the RME the procedure of Hochberg et al. (9) was used except that 100 mM potassium phosphate buffer pH 7.4 was substituted for Tris-HCl. Glycerol and DTT were added to the RME as preservatives. The protein concentration (10) of various extracts ranged from 2.7 to 2.9 mg/ml. The spectrophotometrically determined P_{450} concentration (11) ranged from 0.7 to 0.8 μM. Alkaline phosphatase activity was determined by the method of Garen and Lewinthal (12). One milliunit of activity corresponds to the hydrolysis of 1 nmole of substrate/min./25° at pH 8.0. The cholesterol substrate was prepared according to Tchen (13). Protein kinase was assayed by the method of Corbin and Reimann (14) using histone IIA as the substrate. A unit of activity is defined as the number of pmoles of phosphate transferred to TCA-precipitable material in 10 minutes at 30°. At the end of the incubations a benzene solution (1 ml) containing cholesterol, pregnenolone and progesterone as carriers (0.16 mg of each) was added and the mixture was extracted with three 1 ml portions of benzene. The pooled benzene extracts were blown dry with nitrogen at room temperature and the residues were dissolved in benzene (200 μl). The solutions were spotted on glass plates coated with silica gel G (type 60) and the plates were developed in two dimensions first with the solvent system petroleum ether (b.p. 30-60°)/ether/glacial acetic acid. 65:35:1 (three times to top of plate) and then chloroform,

ethanol, 95:5 in the second dimension. Steroids were visualized with iodine vapor and appropriate sections were scraped from the plates and added to 4 ml of 3a70B scintillation fluid for counting.

Results and Discussion

Rat mitochondrial acetone powder extracts (RME) fortified with an NADPH generating system have the ability to form pregnenolone from cholesterol and thus contain the complete cholesterol desmolase system. We have tested the hypothesis that the acute action of ACTH in accelerating corticoid production may be mediated by protein kinase promoted phosphorylation of a component or components of the cholesterol desmolase system. Indeed such an effect has been observed in a corpus luteum system where pregnenolone formation from cholesterol was significantly stimulated by partially purified corpus luteum protein kinase holoenzyme plus ATP and c-AMP (15). In addition the protein kinase preparation promoted incorporation of [^{32}P] from [^{32}P]-ATP into a component or components of this system. In the present experiments we employed a rat adrenal mitochondrial extract (RME) fortified with an NADPH generating system as the source of cholesterol desmolase and highly purified bovine cytosolic protein kinase isozyme II catalytic subunit as the phosphorylating agent.

The effect of increasing concentrations of RME on pregnenolone formation is illustrated on Figure 1.

The rate of pregnenolone formation was linear with time for at least 30 minutes (results not shown). Since phosphorylation of a component or components of the RME was to be investigated the phosphatase activity of such extracts was assessed. Various RME preparations contained an average of 2.6 milliunits of alkaline phosphatase activity per mg of protein. Phosphatase activity was inhibited by 15 mM L-cysteine or 30 mM DTT. DTT was employed

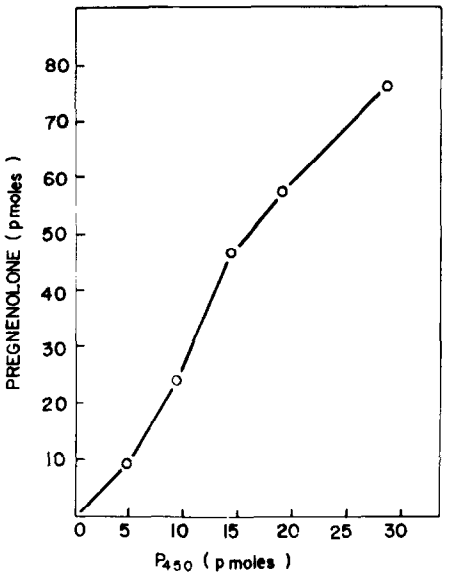

Fig. 1: Effect of increasing concentrations of RME on pregnenolone formation. The incubation mixture (250 µl) contained Hepes buffer pH 7.4 (40 mM); KCl (120 mM); $MgCl_2$ (10 mM); D-glucose-6P (6 mM); NADP (0.6 mM); glucose-6P-dehydrogenase (1U); Trilostane (1 µg); 4-[^{14}C]-cholesterol (sp. act. 91 cpm/pmole) (6 µM); Varying amounts of RME (4.8 to 28.8 pmoles P_{450}) and pH 7.4 potassium phosphate (100 mM) to volume. Incubations were carried out in triplicate in a Dubnoff metabolic incubator at 30° for 15 minutes in air. Results are given as the mean of 3 determinations.

as a phosphatase inhibitor since it did not interfere with the desmolase reaction; L-cysteine inhibits pregnenolone formation (Table 2). Progesterone formation from pregnenolone was suppressed by the addition of Trilostane. The ability of bovine cytosolic protein kinase isozyme II catalytic subunit to phosphorylate histone IIA and RME is illustrated on Table 1.

Histone IIA was used as a substrate to test whether individual components of the complete system caused any inhibition of the protein kinase. The addition of DTT, SCC plus RME or a combination of the three exerted little effect on [^{32}P] incorporation. A low level of phosphorylation (ca. 20 pmoles) above basal level was observed when RME served as the substrate. This may represent specific phosphorylation of a single component of the RME such

Table 1. Protein Kinase Dependent Phosphorylation Using Histone and RME as Substrates

Additions					pmoles ^{32}P incorporated	
Histone	P-Kinase	DTT	SCC	RME	Exp. I	Exp. II
+		+			4.3 ± 0.04	5.2 ± 0.3
+	+				3600 ± 357	-
+	+	+			3950 ± 164	1996 ± 41
+	+		+	+	3677 ± 105	1022 ± 188
+	+	+	+	+	3413 ± 101	1459 ± 109
	+		+	+	24 ± 1.3	15 ± 0.4
	+	+	+	+	22 ± 1.6	15 ± 1.6
	+		+		2.3 ± 0.2	-

The reaction mixture contained [^{32}P]-ATP (5 µCi,74 µM) and sidechain cleavage system (SCC) (150 µl). Cold cholesterol was substituted for radioactive cholesterol and RME was omitted. To this mixture was added as indicated histone IIA (0.6 mg); protein kinase catalytic subunit, Exp. I (5.8×10^3U) (sp. act. 7.2×10^6U/mg); Exp. II (2.1×10^3U) (sp. act. 1.3×10^6U/mg); DTT (8.1 µmoles); and RME (45 µl) containing 24 pmoles of P_{450}. The mixture was diluted to a final volume of 270 µl with 100 mM potassium phosphate buffer pH 7.4. Incubations were carried out in triplicate in a Dubnoff metabolic incubator at 30° for 30 minutes. Results are given ± S.E.M.

Table 2. Effect of Various Additions on Cholesterol Sidechain Cleavage Activity

Additions					pmoles pregnenolone/nmole P_{450}			
None	ATP	P-Kinase	L-Cys	DTT	Exp. I.	Exp. II.	Exp. III.	Exp. IV.
+					1770 ± 34	2787 ± 226		
	+				1460 ± 54	2932 ± 277		
	+	+			1788 ± 168	2840 ± 203		
			+		456 ± 57			
				+	2019 ± 177	2750 ± 102	1538 ± 348	
	+			+	2059 ± 162	2275 ± 335	1477 ± 89	2497 ± 89
	+	+		+	2286 ± 410	2777 ± 236	1700 ± 236	2507 ± 71

The sidechain cleavage system (SCC) (250 µl) contained RME (20-40 µl); Hepes buffer, pH 7.4 (40 mM); KCl (120 mM); $MgCl_2$ (10 mM); D-glucose-6P (6 mM); NADP (0.6 mM); glucose-6P-dehydrogenase (1 U); Trilostane (1 µg); 4-[^{14}C]-cholesterol (sp. act. 91 cpm/pmole) (6 µM) and pH 7.4 potassium phosphate, (100 mM) to volume. Additives when present were added in the following concentrations: ATP (0.32 mM); L-Cys (15 mM); DTT (30 mM); protein kinase, Exp. I. (0.3×10^3 U) (sp. act. 3.6×10^6 U/mg); Exp. II. and III. (0.6×10^3 U) (sp. act. 7.2×10^6 U/mg); Exp. IV. (1.0×10^3 U) (sp. act. 1.3×10^6 U/mg). Incubations were carried out in triplicate in a Dubnoff metabolic incubator at 30° for 15 minutes in air. Results are given ± S.E.M.

as cytochrome P_{450} at a level of 1 pmole [^{32}P]/pmole P_{450} but could also represent nonspecific phosphorylation of other components in the extract. The latter explanation is favored since phosphorylation is not accompanied by a measurable stimulation of pregnenolone formation (Table 2). Furthermore Caron et al. (15) in similar experiments reported that 461 to 494 pmoles of [^{32}P] were incorporated specifically into the corpus luteum system when solubilized P_{450} (23.7 pmoles) was added i.e. over 20 pmoles of phosphate were incorporated per pmole of the P_{450} added. The level of radioactivity used in our experiments was comparable to that used by Caron et al. (15).

The effect of the protein kinase preparation on the ability of RME to convert cholesterol to pregnenolone is shown on Table 2.

No stimulation was observed. The same result was obtained in many experiments using different rat mitochondrial extracts and also a bovine mitochondrial extract (experiments not shown). If the acute stimulation by ACTH of corticoid formation in the adrenal cortex were regulated by phosphorylation of a component or components of the mitochondrial desmolase system one would expect to observe a large stimulation of pregnenolone formation from protein kinase mediated phosphorylation. Stimulation of isolated rat adrenal cells by ACTH results in a 10 to 50 fold increase in corticosterone production (16). Caron et al., observed a 70% increase in pregnenolone formation in the corpus luteum system (15).

The results presented here indicate that the acute stimulation by ACTH of corticoid production by the rat and bovine adrenal is not explicable in terms of protein kinase mediated phosphorylation of a component or components of the cholesterol sidechain cleavage mixed-function oxygenase system.

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