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FAILURE OF ACTH TO MIMIC THE STRESS-INDUCED ACTIVATION OF RAT ADRENOCORTICAL CHOLESTEROL ESTER HYDROLASE *IN VIVO*

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SUMMARY

Rat adrenocortical cholesterol ester hydrolase activity does not respond to the circadian rhythm of the pituitary-adrenal axis under quiescent conditions. However, stress activates this enzyme, producing a greater increase in activity at the high point of the cycle than at the low point. This stress-induced effect requires the presence of the pituitary. It is not mimicked by administration of exogenous porcine ACTH to either hypophysectomized or dexamethasone-suppressed rats, but homogenates of whole rat pituitary tissue do possess substantial stimulatory capacity.

INTRODUCTION

Cholesterol is an immediate precursor for steroids synthesized in the adrenal cortex [1, 2] and one source of this cholesterol is thought to be the pool of cholesterol esters stored in the lipid droplets of the adrenocortical cell [3, 4]. A decline in intracellular cholesterol ester concentration following various steroidogenic stimuli has been widely reported. For example, ACTH administration to intact [5] or hypophysectomized rats [6, 7] induces both a significant ester depletion and a rise in circulating corticosterone. Similar results have been obtained after stress to intact rats [8–10] and addition of ACTH to isolated rat adrenal cells [11].

The rate-limiting step in the steroidogenic pathway is cleavage of the cholesterol side-chain to form pregnenolone [1, 2]. Brownie, Simpson, and associates[12, 13] have presented evidence to suggest that ACTH facilitates the interaction of cholesterol with the cytochrome P-450 side-chain cleavage complex. The total response of this system may also be influenced by the intra-mitochondrial cholesterol available to it, as cholesterol loading in the presence of ACTH increases the rate of steroidogenesis [14, 15].

The provision of cholesterol to the mitochondrion requires hydrolysis of stored cholesterol ester before it can be translocated [7, 14]. Cholesterol ester hydrolase (EC 3.1.1.13), the enzyme responsible for this reaction [16], may itself respond to ACTH via a cAMP-mediated activation [17–20]. The circadian rhythm in basal endogenous ACTH secretion [21, 22] and adrenocortical steroidogenesis [23, 24] in the rat, and the activation of this pituitary-adrenal axis by stress, affords a most appropriate physiological setting in which to investigate any ACTH control of hydrolase activity *in vivo*. We describe here the response of this enzyme in the intact female rat to quiescence and stress at the high and low points of the circadian rhythm and to ACTH administered after hypophysectomy or dexamethasone-suppression.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol oleate, cholesterol [1-^{14}C]-oleate (27 μCi/μmol), oleate and [9,10-^{3}H(*N*)]-oleate (88 μCi/μmol) were obtained from Applied Science Laboratories and further purified by the method of Borgström[25]. Porcine $ACTH_{1-39}$ (Cortrophin) and synthetic $ACTH_{1-24}$ (Cortrosyn) were products of Organon. Fatty-acid-poor bovine serum albumin (BSA) was from Sigma. Corticosterone was from Nutritional Biochemicals. Dexamethasone sodium phosphate (Decadron) was a product of Merck. All other materials were reagent grade or better, and solvents were redistilled before use.

Animals. Female Sprague–Dawley rats, 150–180 g (Holtzman Company), were individually caged and allowed commercial rat chow and water *ad libitum*. Rooms were illuminated from 0600 to 1800 h and kept at 22 ± 1°C. Quiescence was achieved by handling each animal twice daily for at least one week prior to experimentation. In any one experiment, no more than two or three animals were removed from each room. Total elapsed time between room entry and decapitation was less than 20 sec. Stress was accomplished by sham i.p. needle injection. Hypophysectomies were performed transaurally with a Hoffman-Reiter instrument and completeness was determined by random inspection. Dexamethasone (200 μg in 0.1 ml saline) was given i.p. 4 h before experiments. $ACTH_{1-24}$ and $ACTH_{1-39}$ were administered in 0.1 ml gelatin or physiological saline either i.v. or s.c.

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at the times indicated. Control animals received an equal volume of vehicle given by the identical route. Animals were killed by decapitation and blood was collected from the trunk.

Adrenal tissue homogenates. Animals were quickly opened and adrenal glands enucleated *in situ* [26] to obtain adrenocortical tissue free of zona glomerulosa. Tissue was homogenized in a buffer (4 ml/adrenal pair) consisting of 15 mM Tris–HCl, 250 mM sucrose, and 1 mM EDTA, pH 7.4 at 4°C, with three passes of a motor-driven Teflon pestle set for 1800 rev./min. The cytosol fraction was obtained by centrifugation of the homogenate at 105,000 *g* for 60 min. An aliquot of supernatant was then carefully removed from beneath the floating lipid layer by Pasteur pipet.

Cholesterol ester hydrolase assay. The assay for cholesterol ester hydrolase was performed immediately on 0.20 ml aliquots of the 105,000 *g* supernatant (40–100 μg protein) by a double isotope method [18, 27]. Briefly, substrate cholesterol [1-^{14}C]-oleate (4×10^4 c.p.m./assay tube) with cholesterol oleate carrier (500 nmol/tube) in 0.10 ml warmed ethanol was introduced into 4.0 ml of a 100 mM potassium phosphate buffer, pH 7.4 at 37°C, by rapid injection through a 22-gauge needle. After a 5 min sample preincubation at 37°C, 0.20 ml of this buffered substrate emulsion was added to each tube. The reaction was allowed to proceed for 15 min and then terminated with 2.0 ml of an extraction solvent containing unlabelled oleate carrier and [^{3}H]-oleate (3×10^3 c.p.m./tube) to correct for procedural losses. Following addition of 0.05 ml 0.8 N NaOH, extraction, and centrifugation at 1500 rev./min for 15 min, an 0.9 ml aliquot of the upper, aqueous phase was taken into 10 ml of toluene-based scintillation fluid and counted in a Packard Tri-Carb Model 3003. Both ^{14}C and ^{3}H counts were corrected for channel crossover. Enzyme activity was calculated as nmol of oleate formed/min/mg protein. Samples from either individual adrenal pairs or pooled tissue were assayed in triplicate. Reaction blanks consisted of random sample aliquots incubated for the prescribed time and quenched prior to substrate addition. The reaction was linear over the protein concentration range and time period employed. Protein determinations were performed by the method of Bradford[28] using BSA standards.

Serum corticosterone assay. Trunk blood was collected into polypropylene tubes, held on crushed ice for 1 h, and then centrifuged at 2500 *g* for 30 min. Serum was decanted and refrigerated until assay. Corticosterone was measured in a Turner fluorometer by the method of Silber *et al.*[29] using an ethanolic-H_2SO_4 reagent. Rat serum stripped of steroids served as blanks. Samples and standards of authentic corticosterone were run in duplicate.

Whole pituitary homogenate. Rat whole pituitaries were collected at hypophysectomy or obtained from freshly-decapitated animals. Tissue was pooled and homogenized in 0.2 N HCl (0.1 ml/gland) [30]. After standing on crushed ice for 1 h, the homogenates were centrifuged in polypropylene tubes at 8000 *g* for 5 min. The decanted supernatant was stored in liquid N_2 until injection, at which time it was neutralized with aqueous $NaHCO_3$.

RESULTS

Response of adrenal cholesterol ester hydrolase activity to stress at high and low points of the circadian rhythm

The response of rat adrenal cholesterol ester hydrolase to stress induced at the high and low points of the circadian rhythm in adrenocortical activity is shown in Table 1, together with the corresponding levels of serum corticosterone. Enzyme activity was stimulated by stress to a greater extent at 1800 h (163% of quiescent controls) than at 0800 h (149% of controls). Under quiescent conditions, however, despite the 7-fold rise in serum corticosterone from 0800 to 1800 h, the basal activity at the two points was not significantly different. Sampling at 4 h intervals suggests either an absent or very modest circadian rhythm in basal hydrolase activity (data not shown).

Table 1. Effect of stress at the high and low points of the adrenocortical circadian rhythm on cholesterol ester hydrolase activity and serum corticosterone concentration

Treatment	*n*	Cholesterol ester hydrolase Activity†	Cholesterol ester hydrolase % Control	Serum corticosterone μg/dl
0800 h Quiescent	15	2.01 ± 0.06	100	4 ± 2
0800 h Stress	20	2.99 ± 0.05	148	38 ± 3
1800 h Quiescent	12	2.19 ± 0.08	100	29 ± 3
1800 h Stress	20	3.56 ± 0.18	163	69 ± 6

Stress was by sham i.p. needle injection 15 min before decapitation. Data are compiled from two or three experiments. Rat adrenal cholesterol ester hydrolase activities represent $\bar{x} \pm$ SEM from assays performed on individual animals as described in the text. Serum corticosterone concentration was assayed as described in the text and values represent $\bar{x} \pm$ SEM of individual samples determined in duplicate. The difference in mean activity between 0800 and 1800 h is significant at $P < 0.01$ in stressed groups but not significant in quiescent groups. The difference in mean activity between stressed and quiescent groups at each time period is significant at $P < 0.001$. † Activity is expressed as nmol of oleate product formed/min/mg protein at 37°C, pH 7.4.

Table 2. Effect of ACTH or stress on rat adrenal cholesterol ester hydrolase activity and serum corticosterone concentration in hypophysectomized or dexamethasone-treated rats

Treatment	n	Cholesterol ester hydrolase Activity†	% Control	Serum corticosterone μg/dl
Dexamethasone-treated controls	10	1.59 ± 0.06	100	4 ± 1
Dexamethasone + 100 mU ACTH	8	1.57 ± 0.07	99	73 ± 6
Hypophysectomized controls	10	1.60 ± 0.05	100	3 ± 1
Hypophysectomy + 100 mU ACTH	5	1.58 ± 0.04	99	61 ± 5
Hypophysectomy + 0800 h stress	4	1.58 ± 0.04	99	2 ± 1
Hypophysectomy + 1800 h stress	4	1.59 ± 0.05	99	3 ± 1

Hypophysectomy was performed 20–24 h or dexamethasone (200 μg in 0.1 saline i.p.) was injected 4 h before experiment. Stress by sham i.p. needle injection or 100 mU ACTH i.v. in 0.1 ml gel was employed 15 min before decapitation. Adrenal cholesterol ester hydrolase activity and serum corticosterone concentration were determined in triplicate and duplicate, respectively, on individual animals as described in the text. Experimental values represent $\bar{x}$ ± SEM. † Enzyme activity is expressed as nmol of oleate product formed/min/mg protein at 37°C, pH 7.4.

Adrenal cholesterol ester hydrolase activity following stress or ACTH administration in hypophysectomized or dexamethasone-treated rats

The effect of acute stress observed on cholesterol ester hydrolase activity suggests that the response may be due either directly or indirectly to some influence of the pituitary on the adrenal cortex. Data in Table 2 supports this assumption; we found no increase in enzyme activity when acutely (20–24 h) hypophysectomized animals were stressed at either 0800 or 1800 h.

Surprisingly, however, hydrolase activity in hypophysectomized animals was not increased 15 min after the i.v. administration of 100 mU $ACTH_{1-39}$, even though stimulation of steroidogenesis was clearly evident from the rise in serum corticosterone to 61 ± 5 μg/dl (Table 2). Enzyme activity also failed to respond when ACTH was given i.v. to dexamethasone-suppressed rats (4 h), despite a similar rise in serum corticosterone (73 ± 6 μg/dl).

Dynamics of adrenal cholesterol ester hydrolase response to stress or ACTH

We investigated the possibility that sampling at 15 min after ACTH injection was not optimal for the observation of any increase in hydrolase activity. In contrast with our earlier experiments using 100 mU ACTH i.v., we administered 8 U ACTH s.c. to dexamethasone-suppressed animals in order to maximize any measurable change. The time-response curve for enzyme activity (A) and serum corticosterone concentration (B) appears in Fig. 1. The course of steroidogenic response to 0800 h stress in intact rats is also shown for comparison.

The optimum time for measuring peak hydrolase activity after either stimuli was about 10 min; within 20 min activity had returned to control levels. It remained essentially unchanged thereafter for at least 3 h post-injection. Hydrolase activity at this 10 min peak was substantially greater in stressed animals (161% of control) than in those receiving 8 U ACTH

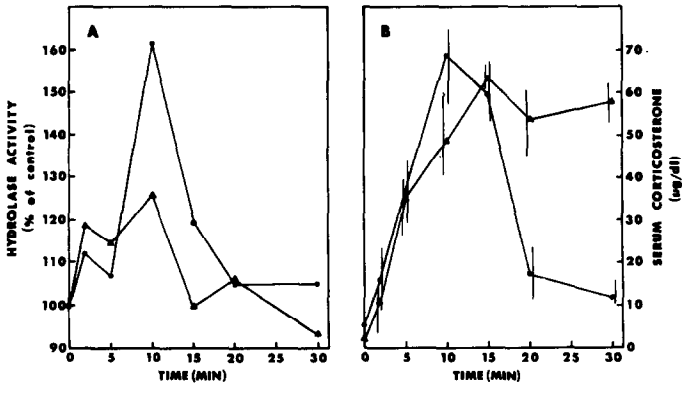

Fig. 1. Time-response curve of (A) adrenal cholesterol ester hydrolase activity and (B) serum corticosterone after stress (●) or s.c. administration of 8 U ACTH (▲) to dexamethasone-treated rats. Animals were given 200 μg dexamethasone i.p. 4 h before experiment. Eight U ACTH made up to 0.1 ml in gel vehicle was injected s.c. Controls received 0.1 ml of vehicle only. Stress was by sham i.p. injection. A group of three animals was killed at each of the indicated times. Each experimental point for hydrolase activity is the mean of triplicate determinations on the pooled glands from each group. The mean coefficient of assay variation was 4.4%. Each experimental point for serum corticosterone concentration is the mean of all values for animals in a group after assay of individual sera in duplicate. Bars denote the range of values observed. Assays were performed as described in the text.

(128% of control). Serum corticosterone following stress also peaked at 10 min and then fell rapidly, in agreement with another study [31], but the level in animals receiving ACTH s.c. remained maximally elevated for at least an additional 20 min. This may reflect a slow, prolonged uptake of ACTH into blood from the injection site, since i.v. administration of ACTH does not provoke an elevated corticosterone level of such lengthy duration. We have observed a similar rise in adrenal intracellular corticosterone in both groups which is rapid and precedes the increase in serum corticosterone.

Dose-response of adrenal cholesterol ester hydrolase activity to ACTH

A dose-response curve for cholesterol ester hydrolase activity and serum corticosterone concentration measured at 10 min after varying doses of s.c. ACTH appears in Fig. 2. Because of the route of administration, relatively large amounts of ACTH were necessary to elicit high steroid output. Seventy mU produced a corticosterone response that was 50% maximal, but at this dose, hydrolase activity was only 102% of dexamethasone-suppressed controls.

Effect on adrenal cholesterol ester hydrolase activity of $ACTH_{1-39}$, $ACTH_{1-24}$, and whole pituitary homogenate administered to hypophysectomized or dexamethasone-treated rats

Table 3 provides data on the effect of various agents at 10 min after administration to hypophysectomized or dexamethasone-suppressed rats. Synthetic porcine $ACTH_{1-24}$ was used to investigate the possibility that the modest effect which porcine $ACTH_{1-39}$ has on hydrolase activity at very high doses (Fig. 2) might result not from ACTH itself, but from small amounts of peptide impurities present in commercial ACTH preparations. However, $ACTH_{1-24}$ closely paralleled the effect of $ACTH_{1-39}$; an 8 U bolus increased hydrolase activity to 118% of control, compared to a 117% increase with 8 U $ACTH_{1-39}$.

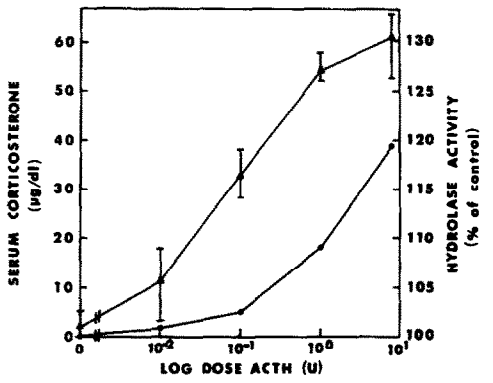

Fig. 2. Dose-response curve for adrenal cholesterol ester hydrolase activity and serum corticosterone concentration 10 min after the s.c. administration of ACTH to dexamethasone-treated rats. Animals were given 200 μg dexamethasone i.p. 4 h before experiment. Varying doses of ACTH made up to 0.1 ml in gel vehicle were injected s.c. 10 min before decapitation. Controls received vehicle only. Each experimental point for hydrolase activity (●) is the mean of triplicate determinations on the pooled adrenals from three animals. The mean coefficient of assay variation was 2.5%. Each experimental point for serum corticosterone concentration (▲) is the mean of all values for animals in a group after assay of individual sera in duplicate. Bars represent the range of values observed. Assays were performed as described in the text.

The failure of porcine ACTH to mimic the activating effect which stress has on cholesterol ester hydrolase, together with the requirement of an intact pituitary for this effect (Table 2), suggested that some other pituitary factor may be either permissive or directly stimulatory. To test this hypothesis we injected a rat whole pituitary homogenate subcutaneously into either hypophysectomized or dexamethasone-suppressed rats. The results in Table 3 show that a quan-

Table 3. Effect of $ACTH_{1-39}$, $ACTH_{1-24}$, and rat whole pituitary homogenate on adrenal cholesterol ester hydrolase activity and serum corticosterone concentration in hypophysectomized or dexamethasone-treated rats

Treatment	n	Cholesterol ester hydrolase Activity†	Cholesterol ester hydrolase % Control	Serum corticosterone μg/dl
Dexamethasone-treated controls	39	1.59 ± 0.04	100	3 ± 1
Dexamethasone + 8 U $ACTH_{1-39}$	25	1.86 ± 0.04	117**	61 ± 3
Dexamethasone + 8 U $ACTH_{1-24}$	6	1.88 ± 0.02	118*	68 ± 6
Dexamethasone + rat whole pituitary homogenate	10	2.19 ± 0.04	138**	16 ± 2
Hypophysectomized controls	10	1.51 ± 0.03	100	3 ± 1
Hypophysectomy + 8 U $ACTH_{1-39}$	6	1.68 ± 0.04	111*	63 ± 5
Hypophysectomy + rat whole pituitary homogenate	4	2.04 ± 0.02	135**	15 ± 4

Dexamethasone (200 μg i.p. in saline) was administered 4 h before experiment. Rat whole pituitary homogenate (10 pituitaries/ml), 8 U $ACTH_{1-39}$, or 8 U $ACTH_{1-24}$ in 0.1 ml gel or saline was injected s.c. 10 min before decapitation. Controls received 0.1 ml vehicle only. Adrenal cholesterol ester hydrolase activity was determined in triplicate on pooled adrenals as described in the text; values represent $\bar{x} \pm$ SEM of results from two or more experiments. Serum corticosterone concentration was determined in duplicate on individual animals as described in the text; values represent the $\bar{x} \pm$ SEM from all animals in a group. † Enzyme activity is expressed as nmol of oleate product formed/min/mg protein at 37°C, pH 7.4. * $P < 0.01$; ** $P < 0.001$.

tity of homogenate capable of inducing only a modest elevation in serum corticosterone (average of 16 μg/dl) nevertheless activated hydrolase activity to 135–138% of control. This was seen in both suppressed and hypophysectomized animals. We were unable to achieve an increased activity of this magnitude with any ACTH dose up to 8 U (Fig. 2).

DISCUSSION

The presence of a circadian rhythm in rat serum corticosterone concentration is well-established [23]. Furthermore, ACTH in rat serum [21] and the rat pituitary [21, 32] exhibit rhythms in approximate synchrony with fluctuating serum corticosterone, suggesting that the pituitary is proximally responsible for this variation in adrenal output. Nevertheless, the amplitude of the circadian corticosterone cycle in the quiescent animal exceeds by several-fold the amplitude in ACTH release [22]. Moreover, at the high point in basal corticosterone output when cholesterol association with the cytochrome P-450 side-chain cleavage system is maximal, stress superimposes an additional 20 μg/dl increase in serum corticosterone [24]. We interpret these data to suggest that ACTH-promoted cholesterol interaction with $P\text{-}450_{scc}$ is not the sole event responsible for the quantitative variation in adrenocortical response. A number of other factors could conceivably play a role. Among them is hormonal control over cholesterol ester hydrolase activity.

The hypothesis that ACTH mediates cholesterol ester hydrolase activation in the rat adrenal cortex *in vivo* derives from observations that (1) ACTH acts to increase the intracellular cyclic nucleotide concentration in the adrenal cortex [33, 34]; (2) the bovine enzyme can be activated by a cAMP-dependent phosphorylation *in vitro* [17–20]; and (3) exogenous ACTH or stress stimulates adrenal cholesterol ester depletion [5–11]. This last point in particular has been repeatly demonstrated. These reports could support a model in which ACTH acts simultaneously to stimulate the flux of non-esterified cholesterol through the steroidogenic pathway—a step perhaps requiring the "rapidly-turning-over" protein of Garren *et al.*[35]—and to increase the cholesterol substrate available for metabolism by activating the hydrolysis of its fatty ester precursors. By promoting an effect at two distinct but cooperative sites, a rise in serum ACTH could effect an adrenal steroidogenic response of a magnitude greater than a single-effect mechanism would predict.

However, our results (Table 1) do not appear to entirely support this hypothesis. Hydrolase activity was increased above basal levels by stress, and to a greater degree at 1800 h than at 0800 h ($P < 0.01$). This may explain in part the capacity of the adrenal for increased steroid output with stimulation. The increase in enzyme activity probably represents activation rather than induction, for cycloheximide does not block stress-associated cholesterol ester hydrolase activation in intact rats [8]. The pituitary either mediates or is permissive for this effect on the hydrolase because its removal eliminated any stress effect (Table 2). However, enzyme activity was not significantly responsive to changes in the basal circadian rhythm of ACTH, as demonstrated by the comparable quiescent activities observed at 0800 and 1800 h (Table 1). Preliminary data from sampling at 4 h intervals in the cycle support this finding (data not shown).

Under conditions of either subcutaneous ACTH administration (in which serum corticosterone is sustained at an elevated level for a period in excess of 30 min) or needle-injection stress (in which corticosterone declines after 15 min), hydrolase activity in both cases displayed a lag phase of about 5 min, rose between 5 and 10 min, and then was deactivated back to the basal level (Fig. 1). There are recent studies which suggest that circulating high density lipoproteins may be a significant source of precursor cholesterol esters for adrenal steroid formation [36–38]. The primary role of cholesterol esters stored within the cell may therefore be to afford a supply of substrate that is readily accessible during the initial period of an adrenal response. The capacity for a rapid response is important because no significant amount of corticosterone is stored within the gland [39]. Our data (Fig. 1) support this interpretation.

The high basal level of hydrolase activity which we and others [19, 20] report suggests the possibility of some counteracting synthetic reaction that would prevent the formation of large quantities of free cholesterol at inappropriate times. Such a mechanism might involve coordinate control over cholesterol esterification catalyzed by acyl coenzyme A: cholesterol acyltransferase (ACAT) (EC 3.1.2.21) [16,40]. Although this enzyme in the adrenal has not received the attention that has been devoted to cholesterol ester hydrolase, there is some evidence that ACAT activity decreases in ACTH-stimulated isolated rat adrenal cells [41]. Two enzymes catalyzing opposing reactions at intermediate rates under basal conditions, although appearing to drive a futile cycle, would be capable of decreasing the response time required for provision of increased free cholesterol by undergoing rapid activation/deactivation. This could be viewed as analogous to the relationship between phosphofructokinase and fructose-1,6-diphosphatase in glucose metabolism. A delayed return of transferase activity to normal after the hydrolase has been deactivated might explain the prolonged net adrenal cholesterol ester hydrolysis that has been reported after stress [42] or ACTH stimulation of hypophysectomized animals [5]. The possibilities implicit in this model require further investigation.

In view of the absence of a modulation in basal adrenal hydrolase activity in the presence of fluctuating ACTH, it was important to determine whether the stress effect on cholesterol ester hydrolase which we

observed *in vivo* was in fact related to ACTH. We were unsuccessful in attempts to mimic stress activation with 100 mU porcine $ACTH_{1-39}$ given intravenously to either hypophysectomized [20–24 h] or dexamethasone-treated (4 h) animals (Table 2). By optimizing our post-injection sampling time from 15 to 10 min (Fig. 1) and increasing the dose of ACTH to 8 U given subcutaneously, a 28% increase in hydrolase activity was observed at the time point when serum corticosterone was maximal. This activation appears to result from the ACTH itself and not from some peptide impurity that might be present in the commercial preparation because this effect is reproduced by an equivalent dose of synthetic porcine $ACTH_{1-24}$ (Table 3). These increases, however, are substantially less than the activation measured following stress (Table 1 and Fig. 1); indeed, commercial ACTH given to dexamethasone-treated rats in amounts capable of elevating serum corticosterone up to about 30 μg/dl elicit no significant response in enzyme activity (Fig. 2).

This finding that the enzyme is unresponsive *in vivo* to pharmacological doses of porcine ACTH differs from the report of Shima *et al.*[43]. However, we believe the discrepancy arises from differences in assay methodology. The isotopic method requires that endogenous cholesterol ester be quantitatively excluded from the 105,000 *g* supernatant samples in order that the predetermined ratio of labelled:cold cholesterol ester added as substrate can be used to calculate activity. Furthermore, the enzyme may hydrolyze unsaturated esters more efficiently than saturated species [44]. In the report by Shima's group, radiolabelled cholesterol palmitate was used without cold carrier at a non-saturating concentration that approximated the amount of residual cholesterol ester already present. The lower level of this endogenous ester which they report in samples from ACTH-stimulated rats as compared with controls, coupled with the likelihood that endogenous unsaturated ester was utilized preferentially, could well account for the greater hydrolysis of labelled substrate observed in ACTH-associated samples.

The failure of porcine ACTH to reproduce the effects of stress on cholesterol ester hydrolase activity (Table 3) suggested the need for a different approach to the problem. We found that the subcutaneous administration of aliquots of rat whole pituitary homogenate stimulated hydrolase activity to 136% of controls. Serum corticosterone concentrations ranged between 7 and 23 μg/dl. The amount of *apparent* bioactive ACTH in these preparations—about 20–50 mU/injection dose—is incapable of eliciting any significant increase in enzyme activity when given in the form of porcine $ACTH_{1-39}$ (Fig. 2). The effect on hydrolase activity is lost if the pituitary homogenate is preincubated with Pronase.

There are at least two possible explanations for the activating effect of rat pituitary homogenate on the hydrolase. Endogenous rat ACTH—either $ACTH_{1-39}$ or the glycosylated intermediate form—may exert an effect on the rat adrenocortical cell of which porcine $ACTH_{1-39}$ is incapable. Or some other non-ACTH pituitary peptide may produce this effect. We are not yet prepared to speculate on the identity of the agent. However, our experiments favor a product of the corticotroph cell, for if a factor released from some other site were involved, those animals which were suppressed with dexamethasone (a corticosterone analog believed to exert its effects primarily on the corticotroph) would have responded to the stress of handling and ACTH injection with an increase in hydrolase activity similar to that seen in non-suppressed intact rats. Our data, however, were similar for both dexamethasone-treated and hypophysectomized animals (Table 3). It is also noteworthy that Studzinsky *et al.* report that increasingly purified ACTH preparations lose their capacity to promote lipid depletion from the zona fasciculata of the human adrenal [45]. Experiments are in progress to identify the responsible pituitary factor and characterize its behavior more completely.

In summary, we conclude from these results that the activity of cholesterol ester hydrolase in the rat adrenal cortex under basal conditions is not significantly affected by the circadian rhythm of the pituitary-adrenal axis, but this cycle does affect the magnitude of the increase in activity following stress. Hydrolase activation is only present within the first 20 min of an adrenocortical response to stimulation, suggesting that other factors must play a role in any sustained elevation of serum corticosterone and/or decline in intracellular cholesterol esters. Commercial porcine ACTH, even at very high doses, fails to quantitatively reproduce this stress-induced enzyme activation, but rat whole pituitary homogenate possesses a substantial stimulatory capacity.

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