ACTH-induced hydrolysis of cholesteryl esters in rat adrenal cells

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Abstract Rat adrenal cortical cells have been prepared by collagenase dissociation of trypsin-treated adrenal tissue. The content and compositions of cholesteryl ester, phospholipid, and triglyceride fatty acids compare favorably with those of undissociated rat adrenal tissue. During 2-hour control incubations of adrenal cortical cells, steroidogenesis was not detected, and the levels of sterol ester, phospholipid, and triglyceride fatty acids were not significantly altered. Incubations with adrenocorticotropic hormone (ACTH) resulted in corticosterone production and significant depletions of sterol ester and triglyceride fatty acids, but not of phospholipid fatty acids. Although all fatty acid esters of cholesterol were hydrolyzed under these conditions, the greatest contributions to the net decrease in sterol esters were by oleate, arachidonate, and adrenate. Incubations with dibutyryl cyclic adenosine monophosphate (0.5 mM) resulted in significantly greater levels of corticosterone production than did ACTH (250 μunits), but the effects on cellular lipids were comparable to those seen with the tropic hormone. This study represents the first demonstration of hormone-induced hydrolysis of sterol esters in an in vitro cell suspension system. The results are discussed with respect to hormone-sensitive sterol ester hydrolase of adrenal cortex, and to the role of endogenous cholesteryl esters in the steroidogenic pathway.

Supplementary key words phospholipids · dibutyryl cAMP · steroidogenesis

It is well documented that a variety of stress conditions or the administration of ACTH to animals results in a substantial decrease in the total lipid content of the adrenal cortex (1–3). A major portion of this decrease is in the esterified cholesterol fraction, which is generally present in high concentrations (4–6) and is contained in numerous cytoplasmic inclusion droplets (7, 8). The hydrolysis of these esters is catalyzed by a sterol ester hydrolase whose activity in vivo is influenced by ACTH (9). Furthermore, it has been reported that, in vitro, the enzyme is activated by cyclic AMP-dependent protein kinase (10, 11), and that activation involves transfer of the terminal phosphate of ATP to the enzyme protein (12, 13).

The enzymatic hydrolysis of adrenal sterol esters has not been considered an obligatory step in the rapid steroidogenic response to ACTH (1–3), since there is a small active pool of free cholesterol for initiation of hormone synthesis (1). However, these stored esters are considered an important source of a continuous supply of the cholesterol substrate required for prolonged steroidogenesis (1–3). This is of even greater significance in view of the recent evidence (14) that endogenous sterol biosynthesis in rat adrenal is completely inhibited under normal conditions.

Reports of changes in adrenal phospholipids and triglycerides in response to stress or ACTH administration have generally been variable (15, 16). Thus, it remains uncertain whether the metabolism of these particular lipids is under direct or indirect hormonal control.

Adrenal cell suspensions have been extensively employed to elucidate various aspects of steroid hormone production (1–3). While this model appears ideally suited for investigation of the turnover and metabolism of sterol esters, such studies to date have been limited (17, 18). Furthermore, the typical marked depletion of adrenal sterol esters observed in response to stress or ACTH administration in vivo has not been demonstrable in adrenal cell suspensions (17, 18).

In the present investigation, adrenal cortical cells have been prepared by proteolytic dissociation of rat adrenal tissue. The content and fatty acid compositions of cellular sterol esters, phospholipids, and triglycerides have been determined and compared to those of intact rat adrenal. Finally, the effects of adrenocorticotropic hormone (ACTH) and dibutyryl cAMP on steroid hormone production and on cellular lipid levels and compositions have been determined.

Abbreviations: ACTH, adrenocorticotropic hormone; dibutyryl cAMP, N^6,O^2-dibutyryl cyclic adenosine 3':5'-monophosphate; CEFA, cholesteryl ester fatty acids; PLFA, phospholipid fatty acids; TGFA, triglyceride fatty acids.

MATERIALS AND METHODS

Materials

Male (CFN) Wistar rats (150–250 g) were obtained from Carworth Farms. Trypsin (Type TLR, 180–250 units/mg), collagenase (Type I, CLS, 150–200 units/mg), and lima bean trypsin inhibitor were obtained from Worthington Biochemical Corp., Freehold, N.J. Bovine serum albumin (fatty acid-poor) was supplied by Sigma Chemical Co., St. Louis, MO. ACTH was purchased from the U.S. Pharmacopeia in ampules with a specific corticotropic activity of 4.81 IU. Dibutyryl $N^6,O^{2'}$-cyclic $3':5'$-adenosine monophosphate (dibutyryl cAMP) was from Calbiochem. Fatty acids, methyl esters, and lipid standards were purchased from Applied Science Corp., State College, PA, and Supelco, Bellefonte, PA.

Preparation of dispersed adrenal cells

Proteolytic dissociation of rat adrenal cortex was accomplished by a combination of the trypsin procedure of Sayers, Swallow and Giordano (19) and the collagenase technique of Kloppenberg et al. (20). All vessels used for cell preparation were either of Teflon or siliconized (Siliclad, Clay Adams, Parsippany, NJ) glass. Animals were housed in individual cages and allowed food and water ad libitum until use. Each rat was killed with a minimum of handling and in a room isolated from other animals to be used in the study. The adrenals from 10–15 rats were pooled and placed in a 25-ml Erlenmeyer flask containing 25 ml of cold Krebs-Ringer bicarbonate buffer, pH 7.4, which contained 0.2% glucose, 0.5% albumin, and 7.65 mM Ca^{2+} (KRBGA buffer). The adrenals were decapsulated, minced, placed in 10 ml of KRBGA containing 0.25% trypsin, and incubated for 20 min at 37°C in a Dubnoff metabolic shaker (90 oscillations/min) under 95% O_2–5% CO_2. The supernatant was discarded, and the remaining tissue fragments were incubated with fresh 0.5% trypsin solution as above.

The tissue fragments from the second trypsin incubation were then incubated five or six times for 20 min each in fresh 10-ml aliquots of KRBGA buffer containing 0.04% collagenase. The supernatants after each incubation were combined and centrifuged at 150 g for 30 min. The packed cells were resuspended in KRBGA buffer containing 0.1% lima bean trypsin inhibitor. A cell count was taken using a hemocytometer, and the preparation was diluted to give a final cell count of $2-4 \times 10^5$ cells/ml.

Aliquots (1.9 ml) of the cell suspension were incubated in 10-ml Teflon beakers after addition of 0.1 ml of saline or saline containing ACTH (10–3000 μunits) or dibutyryl cAMP (0.5 mM). Incubations were carried out for periods up to 2 hr at 37°C in a Dubnoff metabolic shaker (95% O_2–5% CO_2).

Corticosterone assay

At 15-min intervals, 0.2-ml aliquots of the incubation mixture were placed in 15 ml of methylene chloride. Corticosterone was determined by the fluorometric method of Silber, Busch, and Oslapas (21).

Lipid extraction and analysis

For lipid analysis, aliquots (0.5 ml) of the cell suspension were extracted in 20 volumes of chloroform–methanol, according to the method of Folch, Lees, and Sloane Stanley (22).

Prior to separation of the solvent phases of the lipid extract, 0.25 μCi of [1-^{14}C]palmitic acid was added to correct for losses from the extraction, derivatization, and chromatographic procedures. Major lipid classes were separated by silicic acid thin-layer chromatography in a solvent system of hexane–ethyl ether–acetic acid 80:16:4 (v/v). Lipid spots were visualized with Rhodamine 6G and identified by comparison with authentic standards spotted in channels on either side of the same chromatoplate.

Areas corresponding to phospholipids, triglycerides, free fatty acids, and cholesteryl esters were individually scraped into screw-cap tubes. Triglycerides, free fatty acids, and cholesteryl esters were extracted from the silicic acid with diethyl ether–methanol 3:1 (v/v) according to Gartner and Vahouny (23). These, together with the unextracted phospholipid fraction, were derivatized with boron trifluoride–methanol (14% by weight) for 2 hr at 70°C, according to the procedure of Morrison and Smith (24). Under these conditions, all lipid fractions (including unextracted phospholipids) are quantitatively derivatized (23).

Fatty acid methyl esters were determined on a Beckman GC65 chromatograph equipped with a flame ionization detector, and interfaced with a Hewlett-Packard 338OA integrator. Separations were accomplished on a 6-ft glass column (OD 4 mm, ID 2 mm) packed with 10% SP 2340 on 100/120 Chromasorb WAW (Supelco). Column temperature was 180°C and carrier gas flow was 25 ml/min. (internal standard, methyl heptadecanoate, 0.5 μg/μl).

The mean recovery of labeled palmitic acid through the entire procedure was 96.8%. Duplicate analyses of free fatty acids, cholesteryl ester fatty acids, triglyceride fatty acids, and phospholipid fatty acids differed by 5.9 ± 1.0%, indicating a high degree of
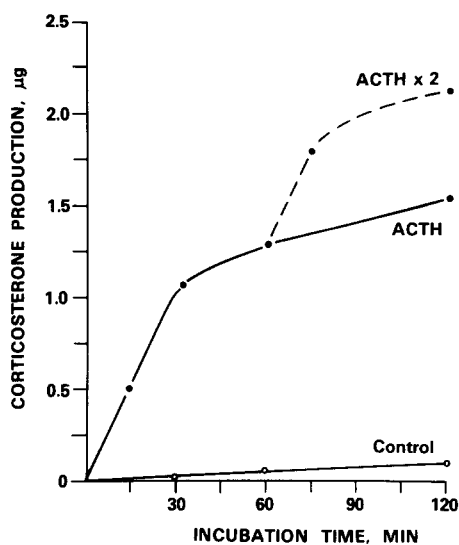
Fig. 1. Corticosterone production by rat adrenocortical cells (2.5×10^5 cells) in the absence (O) and presence (●) of 250 μunits ACTH. Incubations were at 37°C with shaking and under 95% O_2–5% CO_2. The dashed line ●– – –● represents increased corticosterone production by addition of a second dose of ACTH at the time indicated.

reproducibility in the extraction, separation, derivatization, and analysis procedures.

RESULTS

General characteristics of adrenal cell suspensions

Incubations of rat adrenal minces in 0.25% and 0.5% trypsin did not result in the dissociation of intact adrenal cells. However, this trypsin preincubation of tissue was found necessary for efficient dissociation of the tissue with 0.04% collagenase. Successive incubations with collagenase resulted in the continued release of intact cells up to 160 min. of incubation. Although the absolute numbers of cells obtained varied between tissue samples, cell counts (determined by repetitive microscopic determinations) ranged from 1.5×10^5 to 5.0×10^5 cells/adrenal. These yields are comparable to those reported using other techniques (19, 25, 26). Examination of the cells by transmission electron microscopy revealed that over 90% of the intact cells were from the fasciculata region of the adrenal cortex. Viability (as determined by staining with trypan blue) averaged 88.0 ± 1.0%, and was not altered during a 2-hr incubation in the presence or absence of ACTH (10–3000 μunits).

Incubation of adrenal cells with as little as 10 μunits of ACTH resulted in net corticosterone production of 0.19 μg/10^5 cells per 2 hr. This level compares favorably with those from cells produced by

the trypsin disaggregation procedure (19). Furthermore, cells incubated at 37°C for periods up to 2 hr prior to addition of ACTH remained as responsive to the tropic hormone as those to which the hormone was added immediately after preparation. Incubations with progressively higher levels of ACTH (up to 3 munits/ml) resulted in a sigmoid dose-response curve, as has previously been reported (25, 26). The production of corticosterone by 2.5×10^5 cells in the absence and presence of 250 μunits of ACTH is shown in **Fig. 1.** During 2-hr incubations of unstimulated cells, only negligible amounts of steroid hormone were produced. Incubations in the presence of ACTH resulted in rapid corticosterone production which was linear for about 30 min, depending on ACTH concentration, after which the rate of hormone production slowed dramatically. Addition of a second dose of ACTH at this point resulted in an additional and significant increment in corticosterone production. Similar observations have been reported previously (27).

As a further measure of cellular membrane integrity, oxidation of [U-^{14}C]glucose (Amersham/Searle, Arlington Heights, IL) and the insulin sensitivity of dispersed adrenal cells were determined in the presence and absence of 250 μunits of ACTH. In the presence of ACTH alone, corticosterone production and glucose oxidation were 1.1 μg and 1.2 μg/2 hr, respectively. Adrenal cells incubated with 1 milliunit of insulin (Worthington Biochemical Corp.) showed a negligible corticosterone response over control (0.2 μg/2 hr), as has been previously reported by others (25, 26). Oxidation of the labeled glucose was increased to 14 μg during the incubation period. In the presence of both hormones, glucose oxidation was further increased to 23.2 μg/2 hr, and the corticosterone level was 0.7 μg. This small but reproducible decrease in ACTH-induced corticosterone production due to insulin has also been reported earlier (26).

From these studies, it was apparent that adrenal cells obtained by the sequential trypsin and collagenase dissociation procedure retained typical ultrastructural characteristics of fasciculata cells (28), responded predictably to ACTH, and retained insulin sensitivity.

Comparative lipid and fatty acid compositions of adrenocortical cells and intact adrenal tissue

The fatty acid compositions of the major lipid fractions of dispersed adrenal cells and of intact rat adrenals are compared in **Table 1.** The major fatty acid of the cholesteryl esters in both cells

TABLE 1. Fatty acid composition of the major lipid fractions of isolated adrenal cortical cells and intact adrenal tissue

Fatty Acid[a]	Cholesterol Esters		Phospholipids		Triglycerides	
	Cells	Tissue	Cells	Tissue	Cells	Tissue
	% Composition					
14:0	2.2 ± 0.08	2.7 ± 0.5		1.3 ± 0.2	4.7 ± 0.73	2.5 ± 0.3
16:0	12.7 ± 0.68	9.5 ± 0.5	12.7 ± 0.72	10.6 ± 0.52	23.1 ± 0.68	25.1 ± 0.77
18:0	3.5 ± 0.27	3.2 ± 0.40	34.7 ± 1.90	37.6 ± 1.35	13.3 ± 0.80	8.2 ± 1.02
18:1	14.1 ± 0.60	11.2 ± 0.90	10.3 ± 0.65	10.9 ± 0.47	23.8 ± 1.70	31.9 ± 1.76
18:2	5.8 ± 0.12	6.7 ± 0.70	5.9 ± 0.15	5.4 ± 0.10	25.8 ± 1.4	22.2 ± 1.78
22:0	3.6 ± 0.17	3.2 ± 0				
20:4	20.3 ± 0.33	21.0 ± 0.50	29.0 ± 1.40	31.8 ± 1.13	7.5 ± 0.74	4.7 ± 0.79
22:4	13.2 ± 0.42	12.7 ± 1.60		1.0 ± 0.23		
22:5	7.5 ± 0.16	7.9 ± 0.50				
22:6	9.0 ± 0.23	11.2 ± 0.90				

[a] Fatty acids are designated by number of carbon atoms and number of double bonds; tentative identifications were made by comparisons with authentic standards. Values represent means ± SEM where n = 5 for tissue and n = 11 for cell preparations. The major fatty acids in each lipid class are underlined to emphasize major fatty acids in each lipid class. In addition to those fatty acids shown, there were also minor amounts (<2.0%) of 20:0, 20:1, and 20:2 detected in the three major lipid classes.

and tissues was arachidonic acid [20:4(n-6)] with lesser amounts of oleic, palmitic, and the 22-carbon polyunsaturated acids. Overall, the 20- and 22-carbon tetraenoic, pentaenoic, and hexaenoic acids made up approximately 50% of the cholesteryl ester fatty acids (CEFA). This fatty acid distribution is comparable to those reported earlier (6, 29) and indicates that the tissue dissociation procedure does not result in significant changes in the CEFA composition.

The fatty acid composition of adrenal phospholipids differed considerably from that of the sterol esters in that 65–70% of the fatty acids in this fraction were stearic and arachidonic acids. Again, the fatty acid composition of the phospholipids in the cells and whole tissue were comparable and agreed with the pattern of phospholipid fatty acids of rat adrenal tissue reported earlier (30).

The fatty acid composition of the triglycerides was the most variable of the adrenal lipid fractions and has been reported to be subject to the influences of diet, age, and other factors (30). As shown in Table 1, the adrenal tissue triglycerides contained predominantly palmitic, oleic, and linoleic acids. In contrast, adrenal cell triglycerides contained lesser concentrations of oleic acid and higher proportions of stearic acid.

ACTH-induced alterations in adrenal cell lipids

In preliminary experiments, it was found that extreme care was required in animal care, handling, and killing in order to obtain adrenal cortical cells appropriate for studies on lipid metabolism. Unless special care was taken, there were marked depletions of all three lipid classes during cell preparation and incubations in the absence of ACTH. This occurred even though the cells appeared morphologically normal and displayed predictable steroidogenic responses to ACTH or dibutyryl cyclic AMP. Thus, it was found necessary to house animals in individual cages in several animal rooms, and to remove each animal quietly to a separate area for killing. Under these latter conditions, it was possible to obtain reproducible data on adrenal lipid alterations.

As shown in **Fig. 2,** incubation of dispersed adrenal cells (2.5×10^5 cells/ml) for 2 hr at 37°C in the absence of ACTH did not significantly change the preincubation levels of cholesteryl esters, phospholipids, or triglycerides. When cells were incubated with 250 μunits of ACTH or 0.5 mM dibutyryl cAMP, there was a reproducible depletion of CEFA to 65–70% of the initial levels. Under these conditions, there was also a lesser and more variable loss of cellular triglycerides. However, incubations with ACTH or the cyclic nucleotide had no significant effect on the initial level of adrenal phospholipid fatty acids.

Data on various parameters of adrenal cholesteryl ester metabolism are summarized in **Table 2.** In eight separate experiments in which adrenal cell suspensions were incubated for 2 hr in the absence
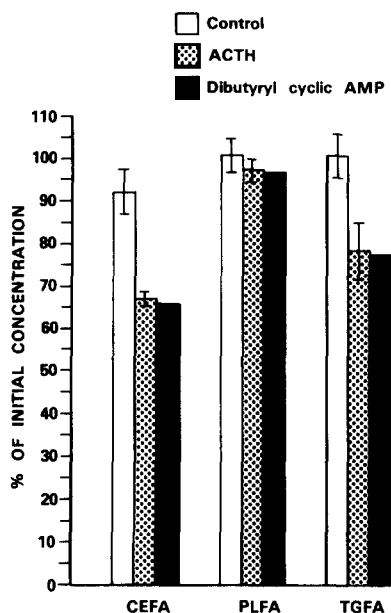
Fig. 2. Changes in adrenal cell lipids during 2-hr control incubations and incubations with ACTH (250 μunits) and dibutyryl cAMP (0.5 mM). Results are expressed as percentages ±SEM of lipid levels in adrenal cells prior to incubation. Values for lipid percentages with dibutyryl cAMP represent means from two experiments. CEFA, cholesteryl ester fatty acids; PLFA, phospholipid fatty acids; TGFA, triglyceride fatty acids.
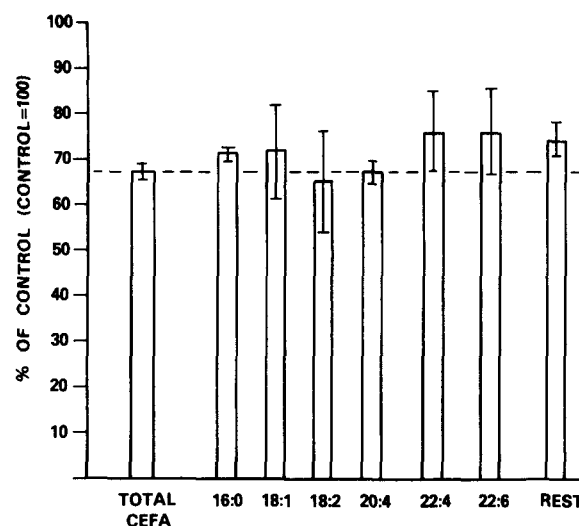
Fig. 3. ACTH-induced hydrolysis of individual fatty acid esters of cholesterol in adrenal cortical cells. Incubations were for 2 hr under 95% O_2–5% CO_2. Values represent means of four studies ±SEM and are expressed as percentages of the values of each ester prior to incubation.

of ACTH, there was no significant corticosterone production, and cholesteryl ester fatty acids were 92.6 ± 5.0% of preincubation levels. Cells exposed to 250 μunits of ACTH for 2 hr produced 0.75 μg of corticosterone, and CEFA was depleted by al-

TABLE 2. Effect of ACTH on corticosterone production, and the content and fatty acid composition of cholesterol esters of adrenal cortical cells

Measurement[a]	Incubation[b]	
	Control	ACTH
Corticosterone, μg	0.0 ± 0.04	0.75 ± 0.21
Cholesteryl ester content		
A. μg	86.0 ± 3.2	62.6 ± 5.1
B. % of 0-time control	92.6 ± 5.0	75.6 ± 1.3
Cholesteryl ester fatty acids, μg[c]		
16:0	8.4 ± 0.7	5.8 ± 0.5
18:0	4.3 ± 0.3	4.1 ± 1.0
18:1	13.2 ± 1.2	8.9 ± 2.1
18:2	6.3 ± 0.6	3.9 ± 0.8
20:4	23.2 ± 0.6	15.0 ± 0.9
22:4	15.6 ± 2.0	11.7 ± 1.4
22:5	4.0 ± 1.0	1.2 ± 0.3
22:6	6.3 ± 1.6	4.7 ± 1.9

[a] All determinations are expressed in terms of amounts in 2.5×10^5 cells.

[b] Incubations were for 120 min at 37°C in 95% O_2–5% CO_2.

[c] Fatty acids are designated by their carbon number and degree of unsaturation. In addition to those shown, other fatty acids in concentrations less than 4 μg/2.5×10^5 cells included 14:0, 20:0, 20:2, 20:3, and 22:0.

most 24 μg to about 75% of the control levels. Although the depletion of sterol esters induced by ACTH was largely accounted for by the esters of palmitate, oleate, linoleate, arachidonate, and the 22-carbon polyunsaturated acids, each of the individual esters of cholesterol contributed to the decrease. As shown in **Fig. 3,** the depletion of each of the sterol esters, calculated as a percentage of their initial concentrations, was statistically comparable to the overall percentage change in the CEFA. Thus, the contributions of the individual sterol esters to the net decrease in CEFA is proportional to the initial concentration of each ester within the cell.

The comparative effects of ACTH and the second messenger derivative, dibutyryl cAMP, on steroidogenesis and adrenal cell lipids are summarized in **Table 3.** Under the conditions of these studies, there was a greater steroidogenic response of adrenal cells to the nucleotide derivative (0.5 mM) than to ACTH. However, with respect to adrenal lipids, responses to incubations with the tropic hormone or dibutyryl cAMP were comparable (see also Fig. 2). In addition, the percentage contribution of individual esters of cholesterol to the net decrease in this lipid fraction due to ACTH or the second messenger were almost identical.

Triglyceride fatty acids were depleted by about 25% whether cells were exposed to ACTH or to dibutyryl cAMP (see also Fig. 2). Approximately 94% of this decrease was accounted for by proportional depletions in palmitic, stearic, oleic, and linoleic acids. There were no significant changes in

the cellular phospholipids in the presence of either ACTH or dibutyryl cAMP (Fig. 2).

DISCUSSION

Recent studies have provided strong evidence for hormone-mediated hydrolysis of adrenal sterol esters. Evidence for a direct effect of ACTH on depletion of adrenal sterol esters in dexamethasone-treated rats was reported by Balasubramaniam et al. (14). Activation of adrenal sterol ester hydrolase by ATP and cAMP and a requirement for protein kinase in the activation process have been demonstrated in preparations of bovine adrenal cortex (10–13). Furthermore, direct evidence that this activation is due to protein-catalyzed phosphorylation of this enzyme has also been reported (12, 13). Despite these and other pieces of evidence (1–3) for the presence of a hormone-sensitive sterol ester hydrolase in adrenal cortex, changes in sterol esters in isolated cell preparations incubated with ACTH have not been previously demonstrated (17, 18). Although the reason for this is not immediately apparent, it is possible that extensive hydrolysis of sterol esters (and triglycerides) had occurred either prior to or during proteolytic dissociation of the tissue. Cellular esterified cholesterol in these earlier studies ranged from 3 $\mu g/10^5$ cells (17) to 18.7 $\mu g/10^5$ cells (18), while in the present study, the levels were 34–48 $\mu g/10^5$ cells (0.17 μg of protein). These latter levels are comparable to those reported for intact cortical tissue of the rat (29).

The physiological role of hormone-induced hydrolysis of adrenal sterol esters and its relationship to steroidogenesis have not been fully elucidated. As shown in the present investigation and in earlier studies (1–3), exposure of adrenal cells to ACTH results is an immediate, but short-lived, steroidogenic response. This occurs at lower ACTH concentrations (5 μunits/ml) than are required to detect sterol ester hydrolysis (250 μunits/ml) in short-term incubations.[1] The short-lived effect of ACTH appears due, in part, to the inactivation or degradation of ACTH by the adrenal cell (31), resulting in cessation of the steroidogenic response. Addition of a second dose of ACTH after cessation of the response to the first dose (Fig. 1.) results in an additional increment of steroid hormone synthesis or release (27). This conversion of available free cholesterol substrate to corticosterone occurs rapidly and appears independent of a continuous supply of cholesterol from other sources, including the sterol ester stores. Thus, cholesterol side-chain cleavage has been considered the rate-limiting step in steroidogenesis (1–3). In contrast to the immediate (24 sec) steroidogenic response to ACTH (32), protein kinase-dependent activation of sterol ester hydrolase in vitro (11–13) and the ACTH-induced hydrolysis of sterol esters in adrenal cells are relatively slow processes.[1] For example, in vitro, sterol ester hydrolysis is not detectable during the initial steroidogenic response to ACTH, but it does continue long after corticosterone production by adrenal cells has ceased.[1] However, in vivo, adrenal steroid hormone production occurs in response to continued ACTH availability, and must therefore be dependent on a continuing supply of substrate for mitochondrial pregnenolone production. It is now apparent that the hydrolysis of stored sterol esters is under direct hormonal control and occurs even when mitochondrial utilization of this cholesterol is blocked by aminoglutethimide (1).

Thus, the activation of adrenal sterol ester hydrolase and the subsequent rate of free cholesterol production and transport to mitochondria may represent a primary limitation to the rate of mitochondrial pregnenolone production. Further studies

TABLE 3. Comparative effects of ACTH and dibutyryl cyclic AMP on corticosterone production and lipids in dispersed adrenal cells

Measurement	Incubations[a]	
	ACTH	Dibutyryl cAMP
	250 μunits	0.5 mM
Corticosterone, μg	0.82 ± 0.05	2.67 ± 0.46
Net decrease in		
A. Cholesteryl ester fatty acids, μg	27.7 ± 0.05	31.0 ± 2.0
B. Triglyceride fatty acids, μg	8.4 ± 0.0	9.1 ± 0.0
C. Phospholipid fatty acids, μg	4.9 ± 1.7	3.2 ± 0.9
Contribution of individual fatty acids of cholesteryl esters to the net decrease, %[b]		
16:0	9.5	8.5
18:0	4.0	3.3
18:1	14.5	14.2
18:2	6.5	5.7
22:0	5.1	3.3
20:4	22.5	22.2
22:4	13.8	12.7
22:5	7.9	6.6
22:6	10.5	11.3

[a] Figures represent means from four experiments with ACTH and two with dibutyryl cAMP; incubation time was 2 hr and with 2.5×10^5 cells/incubation.

[b] In addition to the fatty acids shown, contributions of less than 4% of the net decrease in adrenal sterol esters included 14:0, 20:0, 20:2, 20:3, and 22:0.

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[1] Vahouny, G. V., R. C. Chanderbhan, and R. Hinds. Unpublished observations.

are required to elucidate the mechanisms and control of free cholesterol transport from the lipid droplets (1) to the mitochondria.

It has also been shown in the present study that hydrolysis of adrenal triglycerides occurs in response to incubation with ACTH or dibutyryl cAMP. Although this is the first demonstration of ACTH-induced lipolysis of triglyceride in adrenal cells, Pittman and Steinberg (33) have provided in vitro data to support this finding. These investigators showed that cAMP-dependent lipase activity is demonstrable in the $100,000\,g$ supernatant fraction of rat adrenal homogenates. Furthermore, evidence was provided that this lipolytic activity is separable from the hormone-sensitive sterol ester hydrolase in adrenal cortex (33). The significance of and physiological role of this lipolytic activity have yet to be elucidated.

As is apparent from the data in Tables 2 and 3, the extent of cholesteryl ester hydrolysis, and therefore of free cholesterol release, is in great excess to the amount of corticosterone produced by adrenocortical cells. Although not shown, there is no significant increase in unesterified cholesterol, either in the cells or in the incubation media under these conditions. In an attempt to determine the fate of the released cholesterol, we have recently[2] conducted preliminary studies with adrenocortical cells pre-labeled with [4-^{14}C]cholesteryl esters by injection of labeled cholesterol into the animals 3 days prior to death (35). Incubation of these cells with ACTH results in extensive hydrolysis of [4-^{14}C]cholesteryl esters. However, only 5–10% of the released [4-^{14}C]-cholesterol can be accounted for as authentic corticosterone. The remainder of the radioactivity is recovered as polar compound(s) at the origin of thin-layer silicic acid chromatoplates that have been developed in hexane–ethyl ether–acetic acid 80:16:4. The exact nature of this polar material(s) is currently under investigation.

It is apparent that there are several aspects of adrenal lipid metabolism that require further study. Among these are the mechanism of cholesterol ester uptake by adrenal, regulation of the esterification process, and the possible role of cholesterol arachidonate as a substrate source for prostaglandin synthesis (34). Such studies are now practical with an adrenal cell preparation in which lipid depletions occur in response to ACTH or its suggested second messenger.🔓

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