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ADRENAL CHOLESTEROL ESTERS AS SUBSTRATE SOURCE FOR STEROIDOGENESIS*

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Summary—Adrenocortical cells obtained from tissues of unstimulated rats and which contained a high concentration of endogenous esterified cholesterol, were labeled *in vitro* with unesterified [4-^{14}C]cholesterol, or incubated in the presence of [2-^{14}C]acetate or lipoprotein [4-^{14}C]cholesterol oleate (LP–CE). Incubations were conducted in the absence and presence of ACTH, and changes in the specific radioactivity (SA) of the secreted corticosterone were used to assess the primary sources of cholesterol substrate used for steroidogenesis. Incubations of cells containing [4-^{14}C]cholesterol with ACTH resulted in a marked increase in the output of corticosterone mass, but not of labeled corticosterone. Thus, the SA of corticosterone when cells were incubated with ACTH was only 6.5% of that obtained from cells incubated in the absence of ACTH. During incubations with [2-^{14}C]acetate, the ACTH-induced increase in the output of corticosterone mass was not associated with increased isotope output, and the SA of corticosterone was only 15% of that in control incubations. This dilution was not altered in cells isolated from adrenals of rats treated with 4-aminopyrazalopyrimidine (4-APP), in which increased cholesterogenesis was demonstrable. The uptake, and hydrolysis of LP–CE, and formation of labeled corticosterone was lipoprotein concentration dependent, and was not influenced by ACTH. However, in the presence of ACTH, the SA of the secreted corticosterone was only 4–8% of that in unstimulated cells. The consistent dilution of the SA of corticosterone in ACTH-treated cells in all studies suggest that the large stores of cytoplasmic cholesterol esters in these cells may normally serve as a primary source of the immediate precursor sterol used for steroidogenesis.

INTRODUCTION

The adrenal fasciculata of the rat contains high concentrations of cholesteryl esters, which are contained in cytoplasmic lipid inclusion droplets [1–3]. The cholesteryl ester content of this tissue is measurably reduced during stress, or following treatment with ACTH *in vivo* [1, 4] and during prolonged incubation of adrenal cells with ACTH, cAMP or PGE_2 [3, 5]. This hydrolysis is catalyzed by a neutral sterol ester hydrolase [6], whose activation involves protein phosphorylation by cAMP-dependent protein kinase [7, 8].

These findings imply a potentially important role for endogenous esterified cholesterol in providing the substrate cholesterol for steroidogenesis. However, this relationship has not been directly demonstrated since: (a) activation of the enzyme by cAMP-dependent protein kinase is slow relative to the rapid steroidogenic response of adrenocortical cells to ACTH or cAMP [3, 8]; and (b) only a small level of hydrolysis of esterified cholesterol is required during acute studies to produce a relative excess of substrate cholesterol for the immediate precursor pool [3]. Furthermore, studies conducted *in vivo* are complicated by replacement of esterified cholesterol stores via lipoprotein cholesterol [9].

Other studies have shown that, under specific conditions, where the endogenous cholesteryl esters of the adrenal cortex are depleted either *in vivo* [e.g. 10–13], or by culturing cell preparations in lipoprotein-free media [14–16], either low-density (LDL) or high-density (HDL) lipoproteins could provide substrate cholesterol for steroidogenesis. In these ester-depleted tissues, however, there is also increased cholesterogenesis [10, 11, 13, 14, 16] and reduced steroid output by the tissue [e.g. 13].

The present studies were designed to assess the potential roles of endogenous cholesteryl esters, cholesterogenesis and lipoprotein cholesterol in providing the substrate cholesterol for steroidogenesis in rat adrenocortical cells which normally contained high levels of endogenous esterified cholesterol [3, 5]. An isotope dilution approach was employed in which changes in the SA of secreted corticosterone were assessed in cells prelabeled with unesterified cholesterol, or incubated with either labeled acetate or cholesteryl ester-labeled lipoproteins.

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Abbreviations: ACTH, adrenocorticotropic hormone; PGE, prostaglandin E; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LP–CE, lipoprotein [4-^{14}C]cholesteryl oleate; 4-APP, 4-aminopyrazolo-[3,4-*d*]pyrimidine; SA, specific radioactivity.

EXPERIMENTAL

Materials

Male Wistar rats (Charles River Labs, Wilmington, Mass; 200–250 g) were maintained on standard laboratory chow and water *ad libitum* till use. Lipid standards, obtained from Supelco Inc. (Bellefonte, Pa) and Steroloids Inc. (Wilton, N.H.), were of highest purity. 4-APP was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). ACTH was supplied by the U.S. Pharmacopia (Rockville, Md). Sodium [2-^{14}C]acetate was supplied by Amersham Corp. (Arlington Heights, Ill.), and [4-^{14}C]cholesterol, and [4-^{14}C]cholesteryl oleate were from New England Nuclear (Boston, Mass.).

Methods

Preparation of adrenal cell suspensions. Adrenal glands were routinely obtained from 10–50 unfasted rats following decapitation by the quiescent kill procedure [17]. Where indicated, rats (10 per group) were administered daily i.p. injections of either saline, pH 2.5, or of 4-APP (15 or 50 mg/kg/day in saline, pH 2.5); for 3 days and were killed on the fourth day. Adrenals were minced and placed in pre-oxygenated Krebs' bicarbonate buffer, pH 7.4, containing 0.2% glucose and 0.06% $CaCl_2$ [3]. The preparation of suspensions of adrenal fasciculata cells was as described earlier [3]. Viability, as assessed by trypan blue exclusion, was 94.9 ± 1.4% for all studies. Cells were resuspended in Krebs buffer, pH 7.4, containing 1% lima bean trypsin inhibitor and 0.5% bovine serum albumin.

Prelabeling with [4-^{14}C]cholesterol. Adrenocortical cells (4×10^6 cells/15 ml) were labeled with [4-^{14}C]cholesterol by incubation for 2 h at 37°C under O_2:CO_2 (95:5), following addition of cholesterol (15 μCi) in 25 μl acetone. The suspension was subsequently centrifuged at 150 *g* for 5 min at 4°C and cells were washed twice in Krebs' buffer. In three studies, radioactivity associated with washed cells was 8.0 ± 0.6% of the added [4-^{14}C]cholesterol.

Aliquots of the resuspended prelabeled cells (5×10^5 cells/1.9 ml) were incubated for 60 min at 37°C following addition of 0.1 ml buffer or buffer containing ACTH (0.7 nM). The suspensions were subsequently centrifuged at 150 *g* for 10 min at 4°C, washed once in Krebs' buffer, and recentrifuged. Cells and combined supernatants, along with unincubated prelabeled cells were analyzed as described below. Results are given for one of three studies conducted in triplicate.

Incorporation of [2-^{14}C]acetate. Cell suspensions (5×10^5 cells/1.9 ml) from adrenals of control rats and rats treated with 4-APP were incubated for 60 min at 37°C following addition of 0.1 ml of buffer containing sodium [2-^{14}C]acetate (2 μCi) with and without ACTH. Cells and cell-free supernatants were analyzed as described below. Results are given for one of two studies conducted in triplicate.

Cellular utilization of LP–CE. Rat thoracic duct lymph was subjected to ultracentrifugation at densities of 1.019 and 1.063 g/ml. The $d < 1.063$ g/ml fraction was recentrifuged and dialyzed against three 4 l changes of 0.9% saline–0.3 mM EDTA, pH 7.0. The lipoprotein fraction was characterized by analysis of lipid [3] and apolipoprotein [18] composition.

The purified lipoprotein fraction (300–600 g protein) was labeled *in vitro* with [4-^{14}C]cholesteryl oleate (20 μCi) by the procedure of Brown *et al.*[19]. This was dialyzed overnight against 8 l. of saline–0.3 mM EDTA at 4°C. The preparation used in these studies contained per 100 μl: 5.9 μg total cholesterol, of which 67.0% was esterified; 1.63×10^4 dpm, of which 91.7% was as [4-^{14}C]cholesteryl ester; no measurable triglyceride; and 22 μg protein, of which 60% was as apolipoprotein B (apo B), 22% was apolipoprotein E (apo E) and 18% was apolipoprotein A-1 (apo A-1).

Incubations in the absence and presence of ACTH were conducted for 30 min at 37°C using 1.9 ml cell suspension (4×10^5 cells) and between 25–400 l. of the lipoprotein preparation, adjusted to a final volume of 2.5 ml. Following incubation, duplicate 1 ml aliquots were centrifuged at 150 *g* for 10 min at 4°C. Cell-free supernatants were analyzed as described below. The cells were washed once in buffer and resuspended in buffer containing 10 mg/ml heparin [20]. These were further incubated for 60 min at 4°C prior to recentrifugation and analysis as described below. Radioactivity associated with the cell pellet following incubation with heparin was considered to be internalized [20]. Hydrolysis of LP–CE was taken as radioactivity associated with unesterified cholesterol plus corticosterone minus radioactivity of the lipoprotein unesterified cholesterol radioactivity) which had been internalized. Results are given for one of two studies conducted in triplicate.

Analysis of cellular cholesterol and radioactivity. For all studies, the sedimented cell pellets following prelabeling with cholesterol, or after incubation, were extracted in 10 ml chloroform:methanol (2:1) [21]. The chloroform extract was evaporated to dryness under nitrogen and the residue was taken up in hexane. Major lipid classes were separated by thin-layer silicic acid chromatography in a solvent system of hexane:diethyl ether:acetic acid (80:16:4). Silicic and areas corresponding to unesterified and esterified cholesterol were scraped and extracted three times with diethyl ether:methanol (3:1) [22]. Aliquots of each extract were analyzed for radioactivity by liquid-scintillation spectrometry. Unesterified cholesterol mass was determined by GLC [23] using 5α-cholestane to monitor for recovery. The hexane extract of the esterified cholesterol fraction was evaporated to dryness under nitrogen and the esters were hydrolyzed in 3 ml ethanol containing 3% KOH at 70°C for 60 min under nitrogen. Cholesterol was

extracted with hexane and aliquots were analyzed for radioactivity and mass as described above.

Analysis of corticosterone. One ml aliquots of cell-free supernatants were extracted with 15 ml methylene chloride for analysis of corticosterone [24]. For analysis of labeled corticosterone, aliquots of the cell-free supernatant were extracted in 15 vol of methylene chloride containing 100 μg each of corticosterone, 11-deoxycorticosterone, pregnenolone, progesterone, cholesterol, cholesterol oleate and where appropriate, sodium acetate as carriers. The lower phase was evaporated under nitrogen and steroids were separated by thin-layer silicic acid chromatography in diethyl ether:hexane:acetone (50:30:20). Under these conditions, corticosterone, 11-deoxycorticosterone, cholesterol and cholesterol esters are completely resolved from each other and from other steroids. The silicic acid area corresponding to authentic corticosterone was scraped into scintillation vials for analysis of radioactivity.

Analysis of protein. Protein was determined by the method of Lowry *et al.*[25] using bovine serum albumin as standard.

RESULTS

Utilization of [4-^{14}C]cholesterol by prelabeled adrenocortical cells

Prior to incubation of [4-^{14}C]cholesterol-labeled cells in the absence or presence of ACTH, the cellular unesterified cholesterol fraction was 14.5 ± 0.2% of the total cellular cholesterol and contained 99.0 ± 0.2% of the total sterol radioactivity (Table 1). Following 60 min incubation of these cells in the absence of ACTH, there were insignificant changes in the mass levels of unesterified cholesterol and in the radioactivity or SA of the unesterified cholesterol fraction. However, the SA of the esterified cholesterol increased significantly in the absence of ACTH (Table 1). Corticosterone mass output was low and radioactivity associated with secreted corticosterone was high in unstimulated cells, so that the SA of corticosterone was almost 52-fold that of the cellular unesterified cholesterol.

Following incubation with ACTH, the mass and SA of the cellular unesterified cholesterol fraction was not significantly different from unincubated cells or cells incubated without ACTH. Although the esterified cholesterol mass was 9% lower than in cells incubated in the absence of ACTH, the variability was sufficiently high to preclude statistical significance. The radioactivity and the SA associated with the esterified cholesterol fraction was significantly less than in cells with ACTH and was comparable to the levels in the unincubated controls. There was a marked increase in corticosterone mass output in response to ACTH, but this was not associated with an increase in radioactivity. Thus the SA of corticosterone in ACTH-treated cells was only 6.5% of that with unstimulated cells.

In a separate study, the SA of corticosterone was decreased from 2.9×10^6 dpm/nmol in controls to 7.3×10^4 dpm/nmol in ACTH-treated cells, or a level of 2.5% of that in unstimulated cells.

Incorporation of [2-^{14}C]acetate into cholesterol and corticosterone

Following 60-min incubation of adrenocortical cells (5×10^5 cells/2 ml) with [2-^{14}C]acetate, 0.25% of the label was incorporated into cellular sterols and corticosterone, and this was not altered during incubations with ACTH (0.26%).

As shown in Table 2, the unesterified cholesterol mass in control incubations was 16.6% of the total cellular cholesterol and this was not significantly altered following incubations with ACTH (16.8%). There were also no significant difference between

Table 1. Mass and SA of cellular unesterified and esterified cholesterol and secreted corticosterone during incubations of adrenocortical cells prelabeled with [4-^{14}C]cholesterol

Measurement	Control	− ACTH	+ ACTH
Unesterified cholesterol			
Mass (nmol/mg protein)	75.9 ± 4.7	72.3 ± 7.7	83.9 ± 5.4
Radioactivity (dpm × 10^{-3}/mg protein)	1823 ± 91	1701 ± 137	1781 ± 50
SA (dpm × 10^{-2}/nmol)	240 ± 12	235 ± 7	212 ± 6
Esterified cholesterol			
Mass (nmol/mg protein)	477.7 ± 33.2	451.8 ± 44.6	410.4 ± 10.6
Radioactivity (dpm × 10^{-3}/mg protein)	18 ± 0.8	32 ± 2[a]	18 ± 1[b]
SA (dpm × 10^{-2}/nmol)	0.40 ± 0.02	0.71 ± 0.04[a]	0.44 ± 0.02[b]
Corticosterone			
Mass (nmol/mg protein)	—	0.52 ± 0.02	7.56 ± 0.40[b]
Radioactivity (dpm × 10^{-3}/mg protein)	18 ± 0.8	633 ± 42	602 ± 62
SA (dpm × 10^{-2}/nmol)	—	12,173 ± 808	790 ± 72[b]

Adrenocortical cells were incubated with [4-^{14}C]cholesterol for 120 min at 37°C. Cells were reisolated, washed and aliquots were analyzed for mass and radioactivity associated with the unesterified and esterified cholesterol fractions. Subsequent incubations (5×10^5 cells) were conducted for 60 min in the absence and presence of 0.71 nM ACTH. Cell-free supernatants were analyzed for corticosterone mass and radioactivity. Results are means ± SEM for triplicate incubations in a representative study.

[a] $P < 0.05$ from control; [b] $P < 0.05$ from ACTH.

Table 2. Mass and SAs of cellular unesterified and esterified cholesterol and secreted corticosterone during incubations of adrenocortical cells with [2-^{14}C]acetate

Measurement	− ACTH	+ ACTH
Unesterified cholesterol		
Mass (nmol/mg protein)	72.9 ± 1.4	67.0 ± 1.5
Radioactivity (dpm/mg protein)	516 ± 44	448 ± 6
SA (dpm/nmol)	7.1 ± 0.8	6.7 ± 0.3
Esterified cholesterol		
Mass (nmol/mg protein)	367 ± 2	332 ± 9
Radioactivity (dpm/mg protein)	270 ± 6	290 ± 10
SA (dpm/nmol)	0.74 ± 0.2	0.87 ± 0.05
Corticosterone		
Mass (nmol/mg protein)	1.1 ± 0.06	7.1 ± 0.4[a]
Radioactivity (dpm/mg protein)	4958 ± 24	5123 ± 493
SA (dpm/nmol)	4507 ± 151	720 ± 19[a]

Suspensions of rat adrenocortical cells (5.2×10^5 cells/2 ml) were incubated with [2-^{14}C]acetate (2 μCi) for 60 min in the absence and presence of ACTH. Analysis of cellular sterol mass and radioactivity and of corticosterone are described in the "Experimental" section. Results are means ± SE for triplicate incubations in a representative study.

[a] Significantly different ($P < 0.05$) from control (−ACTH) incubations.

control and ACTH-tested cells with respect to the radioactivity associated with unesterified cholesterol or the SA of this fraction. As in the studies with cholesterol-prelabeled cells, incubations with ACTH resulted in about a 10% lower level of esterified cholesterol than with control cells, but the SA of this fraction was equivalent to that in control cells.

The low corticosterone output by cells incubated in the absence of ACTH was associated with a high level of radioactivity giving rise to a SA of corticosterone which was 635 times that of the total cellular unesterified cholesterol. The increased corticosterone output by ACTH-treated cells, however, was not associated with an increased output of radioactivity, resulting in a decrease in the SA of the hormone to a level only 16.0% of that in the control incubations. In a similar study, the SA of corticosterone was decreased from 3643 dpm/nmol in controls to 92 dpm/nmol with ACTH-treated cells.

Studies with adrenocortical cells from rats treated with 4-APP

Following the 3-day treatment of rats with 15 mg/kg/day of 4-APP, there was a 50% depletion of the esterified cholesterol levels of adrenocortical cells (Fig. 1, left-hand panel), and this was further reduced to 1.9% of controls in rats treated with 50 mg/kg/day 4-APP. Cellular unesterified cholesterol levels, however, were comparable in the three experimental groups. The steroidogenic responses of the cells from the three groups of rats during incubation with ACTH (60 min) are shown in Fig. 1 (right-hand panel). These responses closely paralleled the levels in cellular esterified cholesterol in the three groups.

Incorporation of [2-^{14}C]acetate into total sterol and steroids (unesterified and esterified cholesterol and corticosterone) was not significantly altered in adrenal cells from rats treated with lower dose of 4-APP, compared to cells from control animals (Fig. 2). With cells from animals treated with the higher dose of 4-APP (in which endogenous esterified cholesterol was essentially depleted), there was an almost 2-fold increase in acetate incorporation into sterols and steroids. Under no conditions, however, was acetate incorporation influenced by the presence of ACTH.

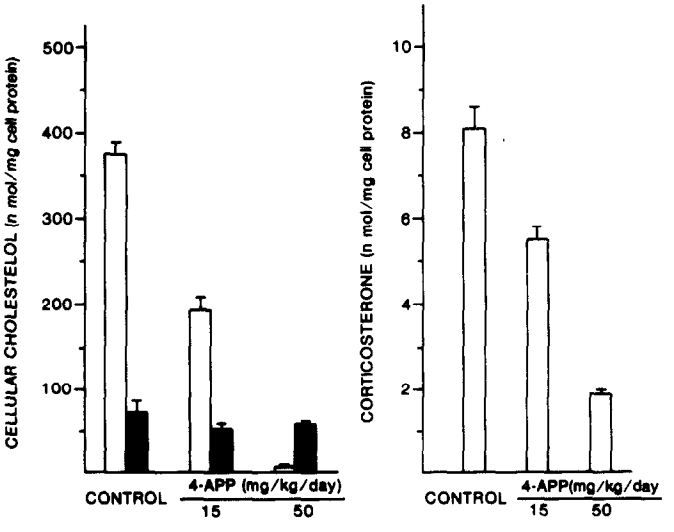

Fig. 1. Levels of cellular unesterified and esterified cholesterol (left-hand panel) and ACTH-induced corticosterone release (right-hand panel) by adrenocortical cells isolated from rats treated with saline or 4-APP. Rats were treated for 3 days with saline or with 15 or 50 mg/kg/day of 4-APP. Preparation of adrenal cell suspensions, incubation conditions and analysis are described under in the "Experimental" section. *Left-hand panel*, cellular esterified (□) and unesterified (■) cholesterol levels in nmol/mg cell protein; *Right-hand panel*, corticosterone production (nmol/mg protein) during 1 h incubation of cells with ACTH. Figures represent means ± SEM for four incubations.

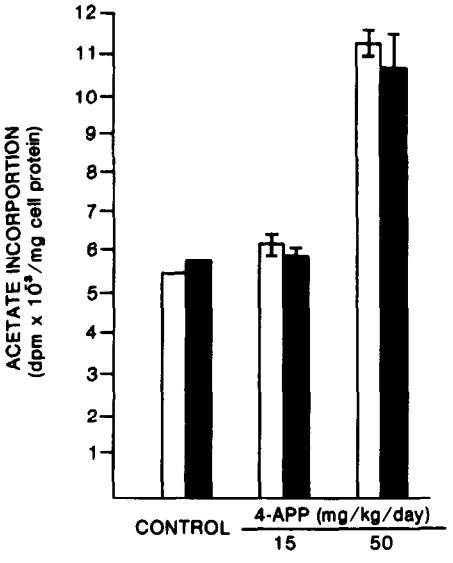

Fig. 2. Total [2-^{14}C]acetate incorporation into unesterified and esterified cholesterol and corticosterone of adrenals cells prepared from control rats and rats treated with 4-APP. Incubations were conducted in the absence (□) and presence (■) of ACTH. Conditions are as given in Fig. 1 and the "Experimental" section.

With adrenocortical cells from rats treated with the lower dose of 4-APP, the SA of corticosterone in the absence of ACTH was 5303 ± 25 dpm/nmol. This was decreased to 656 ± 61 dpm/nmol (12.4 ± 0.61 of control) in the presence of ACTH. In studies with cells from rats treated with a high dose of 4-APP, there was no measurable corticosterone mass output in the absence of ACTH and the comparison of SA in the absence and presence of ACTH was not possible.

Utilization of lipoprotein labeled with [4-^{14}C]cholesteryl oleate

Adrenocortical cells from untreated, quiescent-killed rats were incubated in the absence and presence of ACTH and with four concentrations of lipoprotein cholesteryl ester (LP-CE) ranging from 2.6 to 41.0 nmol esterified cholesterol. As shown in Fig. 3 (left-hand panel), internalization of LP-CE was proportional to the concentration in the incubation media, and was not affected by ACTH. Thus, the mean value for internalization of LP-CE in the absence of ACTH was $15.7 \pm 0.6\%$ of that in the incubation media, and in the presence of ACTH, internalization of LP-CE was $14.3 \pm 0.6\%$ of that in the incubation media. Hydrolysis of the internalized cholesteryl oleate appeared to be saturable at the higher levels of incorporated LP-CE (Fig. 3, right-hand panel), and this was also unaffected by ACTH.

Incorporation of the [4-^{14}C]cholesterol of the LP-CE into corticosterone in the absence or presence of ACTH is shown in Fig. 4 (left-hand panel). With increasing concentrations of LP-CE in the incubation media, there was a progressive, non-linear increase in corticosterone radioactivity which was not affected by ACTH. As shown in Fig. 4 (right-hand panel), baseline corticosterone mass output in the absence of ACTH was low and was not influenced by media LP-CE concentrations. In the presence of ACTH, there was a marked increase in corticosterone mass output, and except at the highest concentration, this was also unaffected by media LP-CE. Thus, the SA of secreted corticosterone in the absence of ACTH was directly proportional to the increased incorporation of radioactivity with increasing levels of media LP-CE (Fig. 5). In the presence of ACTH, increased corticosterone mass associated with the same levels of radioactivity as in unstimulated cells resulted in a marked decrease in corticosterone SA.

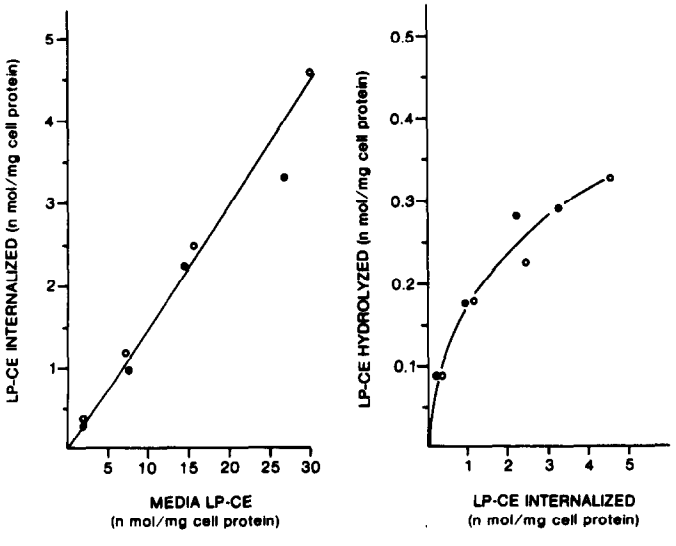

Fig. 3. Internalization (left-hand panel) and hydrolysis (right-hand panel) of LP-CE by rat adrenocortical cells. Incubations (30 min) were conducted in the absence (○) and presence (●) of ACTH.

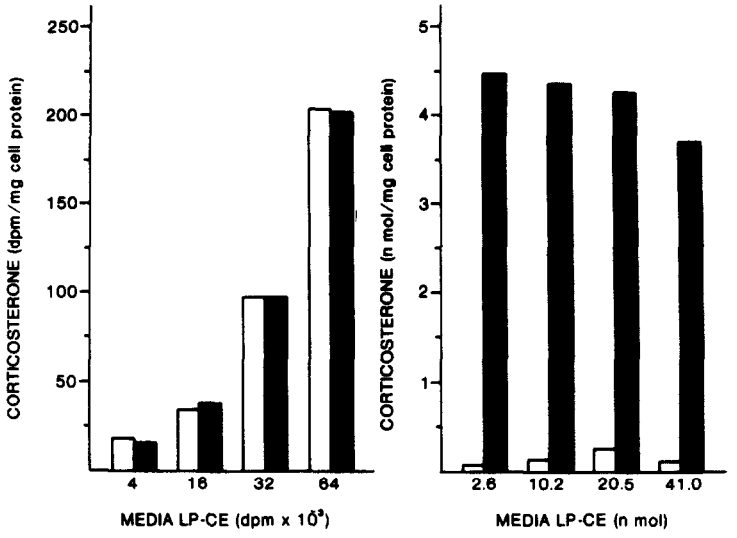

Fig. 4. Incorporation of LP-CE into corticosterone (left-hand panel) and the effect of LP-CE concentrations on corticosterone output by rat adrenocortical cells (right-hand panel). Incubations were carried out in the absence (□) and presence (■) of ACTH.

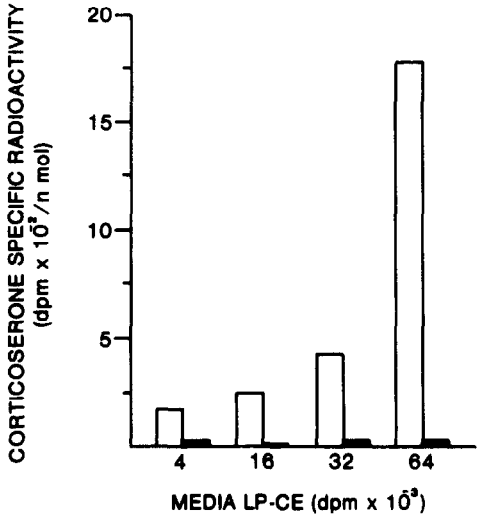

Fig. 5. SA of corticosterone during incubations in the absence (□) and presence (■) of ACTH at varying levels of extracellular LP-CE.

DISCUSSION

There have been only limited attempts to distinguish the relative importance of direct and indirect sources of the cholesterol which are utilized as an immediate precursor for mitochondrial pregnenolone production and ultimate formation of secreted steroids in the adrenal cortex. Current evidence suggests that both circulating LDL and HDL can provide cholesterol to the adrenal, albeit via different cell receptor systems and mechanisms of internalization of the cholesterol [e.g. 10–16]. Furthermore, there is evidence that, in tissues depleted of endogenous cholesteryl ester stores, lipoprotein cholesterol can represent a direct source of substrate to the mitochondrial precursor pool [13, 14, 16].

The role of cholesterogenesis as a substrate source for mitochondrial pregnenolone synthesis is probably of little physiological importance in rat adrenal cortex [13], since even under conditions where cholesterogenesis is derepressed, the increased synthesis of sterol does not appear to provide sufficient substrate for ACTH-induced steroidogenesis [13, 26].

The importance of endogenous cholesteryl esters of rat adrenal cortex as a storage site for the immediate cholesterol requirements of mitochondria has been implied in a variety of *in vivo* and *in vitro* studies [1–8]. Direct evidence, however, has been difficult to obtain since the levels of endogenous cholesteryl esters are in great excess of the amounts of cholesterol required for steroidogenesis in acute studies [3, 5]. Thus, as little as 3% hydrolysis of this ester pool, which is difficult to determine by mass analysis, could provide excess cholesterol required for the acute induction of steroidogenesis.

An attempt has been made to prelabel endogenous cholesteryl esters of adrenal cortex *in vivo*, as described by Ichii *et al.*[27], in order to determine the direct contribution of this storage pool to the cholesterol precursor poor for steroidogenesis. This approach, however, was not useful since only relatively low levels of labeled cholesterol were incorporated by adrenocortical tissue and this was distributed between both unesterified and esterified cholesterol of the isolated cells.

As an alternative approach in the present studies, the cholesterol of adrenocortical cells has been labeled by a variety of approaches, and the SA of secreted corticosterone in unstimulated cells has been taken as a measure of labeling of the cholesterol

precursor pool for steroidogenesis. A comparison of the SAs of secreted corticosterone in these cells with that from cells incubated with ACTH has been taken as a measure of the contribution of unlabeled sources of cholesterol to the cholesterol precursor pool.

In studies with adrenocortical cells prelabeled with unesterified [4-^{14}C]cholesterol, almost 99% of the incorporated radioactivity was associated with cellular unesterified cholesterol, whose SA was about 600 times that of the cellular esterified cholesterol. Incubation of these cells in the absence of ACTH resulted in low baseline levels of corticosterone mass output and a high level of labeled corticosterone output. This represented almost 27% of the total cellular radioactivity (cholesterol, cholesteryl ester and corticosterone), giving an SA for corticosterone almost 52 times that of the total cellular unesterified cholesterol. This suggested that the immediate precursor pool of cholesterol for steroidogenesis is small relative to total cellular cholesterol, and must have a high SA.

Incubation of these cells with ACTH showed that the increased mass of secreted corticosterone was not accompanied by increased flux of radioactivity. The SA of corticosterone under these conditions was only 6.5% of that obtained in the absence of ACTH, attesting to the marked dilution of the labeled cholesterol precursor pool by an unlabeled or poorly-labeled source of cholesterol.

Studies on acetate incorporation into sterols and corticosterone were consistent with the general concept that, under conditions where alternative sources of substrate cholesterol are available, cholesterogenesis is probably of lesser importance in providing the steroid substrate. As reported earlier [13], acetate incorporation into cells from unperturbed rats is small and not influenced by ACTH. Furthermore, during pharmacological depletion of adrenal esterified cholesterol [13], the increase in cholesterogenesis was clearly unable to support the substrate requirement for ACTH-induced steroidogenesis.

The lipoprotein fraction (density 1.019–1.063 g/ml) used in the present studies, was clearly a mixed fraction containing both apo B of LDL and some apo A-1 and apo E, typical of HDL. It was not considered crucial to purify this fraction further, since the adrenal cortex can utilize the cholesteryl esters of either lipoprotein, albeit by different cellular receptor mechanisms, and it was not the primary aim of these studies to distinguish between these receptor-mediated pathways. Thus, emphasis has only been placed on the extent of uptake and hydrolysis of the LP-CE, incorporation of the cholesterol label into corticosterone.

These studies suggested that the uptake and hydrolysis of LP–CE, and the incorporation of cholesterol radioactivity into corticosterone were largely independent of the presence of ACTH. These data are entirely consistent with those recently reported [13] in which adrenal cell suspensions were incubated with serum LDL or HDL labeled with either esterified or unesterified cholesterol.

However, although the absolute level of labeled corticosterone was not affected by ACTH, there was an increase in secreted corticosterone mass, and this was only decreased by 12% at the highest extracellular concentration of LP–CE.

The results of the present studies, when taken in context with those reported previously [1–8], suggest that under normal physiological conditions in which cholesteryl ester content is high, the cholesterol of this storage site may contribute a large proportion of the precursor cholesterol utilized for steroidogenesis.

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