We have reported previously that expression of the human apolipoprotein E (apoE) gene in mouse Y1 adrenocortical cells suppresses basal and adrenocorticotropic (ACTH)-stimulated steroidogenesis. To understand the mechanism of this suppression, we have examined the integrity of cAMP regulated events required for adrenal steroidogenesis. Fast and deleterious responses to ACTH or cAMP are suppressed in Y1 cells which express apoE (Y1-E cells) as compared with parental Y1 cells. Acute morphologic changes in response to cAMP and acute induction of steroidogenesis by cAMP are suppressed in the Y1-E cell lines. Constitutive expression of P450-cholesterol side chain cleavage enzyme mRNA, the rate-limiting enzyme in steroid hormone synthesis, is reduced up to 11-fold in the Y1-E cell lines. The level of mRNA encoding P450-cholesterol side chain cleavage correlates directly with the reduction in basal steroid production observed in the individual Y1-E cell lines. Expression of P450-11β-hydroxylase mRNA, although readily detectable in Y1 parent cells, is absent or reduced in the Y1-E cell lines. Inhibition of cAMP-regulated gene expression is not restricted to genes required for steroid synthesis, since cAMP induction of ornithine decarboxylase mRNA is also inhibited in the Y1-E cell lines. These data indicate that suppression of steroidogenesis in Y1-E cells is due, at least in part, to inhibition of cAMP-regulated gene expression. These effects are not due to a defective cAMP-dependent protein kinase, since kinase activity in vitro and activation in vivo are unaltered in the Y1-E cell lines. These results suggest that expression of apoE in Y1 cells blocks cAMP-mediated signal transduction at a point distal to activation of cAMP-dependent protein kinase.

Apolipoprotein E (apoE) is an important modulator of cholesterol homeostasis in vivo. Unlike most apoproteins which are synthesized only in the liver and intestine, apoE is also synthesized in a number of peripheral tissues (1). Although the role of apoE in these tissues is unclear, apoE may facilitate local redistribution of cholesterol among cells within a tissue or play a role in intracellular cholesterol metabolism (1, 2). ApoE is particularly abundant in steroidogenic tissues; apoE mRNA concentrations are similar in liver and adrenal glands from both monkeys and rats (3, 4), and in human and monkey adrenal tissue apoE is synthesized at a relative rate equal to, or greater than, that observed in the liver (1, 2). Adrenocortical cells in vivo, and cultured adrenal cells in vitro, utilize cholesterol for steroid hormone synthesis. In vivo studies suggest a direct correlation between rat adrenal apoE expression and total cell cholesterol, and an inverse relationship between apoE expression and adrenal steroidogenesis (4). The modulation of adrenal gland apoE expression by agents that alter steroidogenesis and cholesterol metabolism may reflect a role for apoE in some aspect of these processes.

Production of steroid hormones in the adrenal gland is regulated by the peptide hormone adrenocorticotropic (ACTH). ACTH works through specific cell surface receptors to activate adenylate cyclase and increase intracellular cAMP (5). Most, but possibly not all, of the effects of ACTH are thought to occur via this second messenger, through the action of a cAMP-dependent protein kinase (5). These responses include both acute and chronic effects on steroid hormone production. Acute effects are characterized by changes in cell shape and mobilization and transport of cholesterol from storage sites to the inner mitochondrial membrane, the site of cholesterol side chain cleavage (6, 7). These acute responses result in a rapid increase in steroid hormone production presumably due to increased availability of cholesterol substrate (5). ACTH also acts in a chronic manner to maintain optimal levels of P450-steroid hydroxylases, including the rate-limiting enzyme for steroid synthesis, P450-cholesterol side chain cleavage (SCC) (8). Prolonged deprivation of ACTH in vivo, or in vitro, results in decreased levels of these hydroxylases, which can be reversed by addition of ACTH or cAMP (8).

We have reported previously (9) that expression of the human apoE gene in mouse Y1 adrenal cortical cells results in a dramatic suppression of both basal and ACTH-induced steroidogenesis. We report here that this suppression is due, at least in part, to inhibition of cAMP regulated gene expression, including constitutive expression of the steroid hydroxylase genes, SCC, and P450-11β-hydroxylase (11β-OHase). This inhibition is not the result of an altered cAMP-dependent protein kinase, since protein kinase activation appears to be normal in the Y1-E cell lines. These results suggest that expression of apoE in Y1 cells blocks cAMP-mediated signal transduction at a point distal to the activation of cAMP-dependent protein kinase.

MATERIALS AND METHODS

Cell Culture—The Y1 adrenal cell line was obtained from American Type Tissue Culture. The human apoE transfectant subclones (Y1-E cell lines) and the Y1-neo cell line (a control cell line transfectad only with the neomycin resistance gene) have been described previously.
(9). Cells were maintained in Ham’s F-10 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 12.5% (v/v) heat-inactivated horse serum, and 2.5% (v/v) heat-inactivated fetal calf serum. Stock cultures of the Y1-E cell lines were maintained in the presence of 100 μg/ml (active form) G418 sulfonate (geneticin; Gibco). For experiment-influencing fluorogenic (AMP-dependent) measurements, Ham’s F-10 was supplemented as above, except that the serum concentration was reduced to 5% horse serum and 2.5% fetal calf serum. Ham’s F-10 and dibutyryl cyclic AMP (Bt2cAMP) were obtained from Sigma; all additional cell culture reagents were obtained from GIBCO.

mRNA Analysis and Quantification—For Northern blot analysis, 15 μg of total RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to Nytran (Scheicher and Schuell), cross-linked with ultraviolet light (Stratalinker), and hybridized to the indicated cDNA probe. cDNA probes were prepared by random priming in the presence of [α-32P]dCTP (Amersham Corp; 800 Ci/ mmol). For dot blot analysis, the indicated amount of total RNA was blotted onto Nytran, washed twice with 1.5 M NaCl, 0.15 M sodium citrate and cross-linked with UV light (Stratalinker). The rat glyceraldehyde phosphate dehydrogenase cDNA probe was a generous gift of K. Marcu, State University of New York at Stony Brook, the ornithine decarboxylase cDNA probe (pODC-2) (10) was generously provided by P. Blackshear, Duke University, and the clones pSCC-1.8 and pl1β-OHase-1.2 (11) were the generous gift of K. Parker, S. Kirkman, and D. Rice, Duke University. The DNA-excess solution hybridization assay for human (12) and mouse (13) apoE mRNAs were done as described previously.

CAMP-dependent Protein Kinase Activity—Confluent cell monolayers (100-mm2 dish) were rinsed twice with cold 20 mM Tris, pH 8.0, 250 mM sucrose, 1 mM MgCl2, and collected in 1 ml of 50 mM Tris, pH 8.0, 4 mM dithiothreitol, 5 μg/ml leupeptin, 5 μg/ml aprotinin using a cell scraper. The cell suspension was transferred to a Dounce homogenizer, homogenized with 15 strokes of a tight pestle, and centrifuged at 100,000 X g for 60 min at 4 °C. CAMP-dependent kinase activity was assayed in a total volume of 50 μl containing 100 μg of histone H1 (Sigma), 10 mM MgSO4, 20 mM MES, pH 6.5, 10 mM dithiothreitol, 150 μg of bovine serum albumin, 0.5 mM isobutylmethylxanthine, 5 μM NaF, 20 μM [32P]ATP, (400–500 cpm/μmol; Amersham Corp.) and the indicated concentration of cAMP (Sigma). Reactions were initiated by the addition of 10 μl of S100 supernatant and incubated for 5 min at 30 °C. Twenty μl of each sample was spotted onto Whatman phosphocellulose filter discs, filters were batch-washed (10 ml/sample; 15 min/wash) with two changes of 0.85% phosphoric acid and rinsed once with water and once with absolute EtOH. Radioactivity incorporated into H1 histone was determined by liquid scintillation spectrometry.

Miscellaneous—Fluorogenic steroids were measured as described (14) using a modification of the method of Kowal and Fielder (15). RNA was prepared by the guanidine isothiocyanate ethanol precipitation method (16). Cell protein was determined by the method of Bradford (17), using a kit from Bio-Rad.

RESULTS

Acute Responses to cAMP Are Suppressed in the Y1-E Cell Lines—We have demonstrated previously that expression of the human apoE gene in mouse Y1 cells suppresses basal and ACTH or cAMP-stimulated steroidogenesis relative to parental Y1 cells or a transfected control cell line (Y1-neo) (9). To investigate the mechanism of this suppression, we have examined the integrity of several CAMP-regulated events required for steroidogenesis. ACTH, via cAMP, exerts both acute and chronic effects on adrenal steroidogenesis. The acute response is characterized by a rapid increase in steroid production (5) and is accompanied by distinct morphologic changes such as cell retraction and rounding (18). To determine if this acute response is altered in the Y1-E cell lines, we treated Y1-neo, Y1-E12, and Y1-E16 cells with Bt2cAMP and observed their morphology by phase contrast microscopy. As seen in Fig. 1, treatment of Y1-neo cells with Bt2cAMP results in dramatic morphologic changes characterized by cell retraction and rounding. In contrast, treatment of Y1-E cells with Bt2cAMP for either 4 (data not shown) or 24 h (Fig. 1), has little or no effect on the morphology of the Y1-E12 or Y1-E16 cell lines. When other Y1-E cell lines were examined, morphologic responses were found to be either absent (Y1-E1 and Y1-E10) or blunted (Y1-E2 and Y1-E15) (data not shown). Treatment of cells with ACTH produced results identical to those observed with Bt2cAMP (data not shown).

Steroid Hydroxylase Gene Expression Is Suppressed in the Y1-E Cell Lines—ACTH is required for maintenance of normal levels of the major steroid hydroxylases in the adrenal cortex (8). In Y1 cells these enzymes include SCC, expressed in all steroidogenic tissues, and 11-β-OHase, an adrenal-specific enzyme required for the synthesis of corticosteroids. Since SCC catalyzes the rate-limiting step in steroidogenesis, conversion of cholesterol to pregnenolone, reduced expression of SCC could account for the reduction in steroid synthesis we observe in the Y1-E cell lines. To determine if steroid hydroxylase gene expression is altered in Y1-E cells, we examined constitutive expression of SCC mRNA by Northern blot analysis. As seen in Fig. 2, compared with Y1-neo cells, expression of SCC mRNA is dramatically reduced in all the Y1-E cell lines examined. Quantification of SCC mRNA levels by dot blot analysis (Fig. 3 and Table II) shows that expression of SCC mRNA in individual Y1-E cell lines is reduced to 9–61% of the level observed in the Y1-neo cell line. This reduction in SCC mRNA expression correlates with the reduction

### Table I

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Bt2cAMP</th>
<th>Y1-neo</th>
<th>Y1-E12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>36</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>148</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>496</td>
<td>25</td>
</tr>
</tbody>
</table>

**Fig. 1.** Morphologic changes of Y1-neo, Y1-E12, and Y1-E16 cells in response to cAMP. Cells were treated for 8 h with or without 2 mM Bt2cAMP as indicated and photographed by phase contrast microscopy (×20 magnification).
Comparison of the fold reductions of SCC mRNA and basal steroidogenesis is reduced 12-fold. Likewise, SCC steroid synthesis (Table I) suggests that reduced constitutive expression of other steroid hydroxylase genes is suppressed in the Y1-E cell lines, we examined the expression of 11β-OHase mRNA by Northern blot analysis (Fig. 2). 11β-OHase is required for production of the final steroid product of Y1 cells, 11β, 20α-dihydropregesterone. In the absence of 11β-OHase, Y1 cells produce 20α-dihydropregesterone, also detectable in the fluorogenic steroid assay used in Table I. As seen in Fig. 2, although 11β-OHase mRNA is easily detectable in Y1-neo cells, expression is reduced to below detectable levels in all but one Y1-E cell line. In this cell line, Y1-E15, expression of 11β-OHase is reduced approximately 2-fold. The Y1-E15 cell line produces the lowest level of apoE mRNA of the clones we have examined and is the least inhibited in steroidogenesis (Table II).

In Y1 cells maintenance of basal steroidogenesis is probably dependent, at least in part, on the relatively high ACTH levels found in horse serum (21). Expression of SCC and 11β-OHase mRNA above constitutive levels can be induced by incubating cells for 12-48 h in cAMP or an activator of adenylyl cyclase such as forskolin. To determine if cAMP induction of SCC and 11β-OHase mRNA is altered in the Y1-E cell lines, we incubated cells in Bt2cAMP for 24 h and analyzed mRNA expression by Northern blot analysis. As seen in Fig. 4, SCC mRNA is induced 2- to 3-fold in Y1 and Y1-neo cells and in all the Y1-E cell lines examined. The modest increase in SCC mRNA in the Y1-E cell lines corresponds to the 2- to 3-fold stimulation of steroidogenesis observed in response to cAMP (see Table III). These results demonstrate that although constitutive expression of SCC mRNA is suppressed in the Y1-E cell lines, SCC can respond to cAMP with a fold induction comparable with that observed in Y1 or Y1-neo cells. Nonetheless, even after induction with cAMP, expression of SCC in the Y1-E cell lines is still greatly suppressed compared with the control cell lines. When cAMP stimulation of 11β-OHase mRNA was examined (Fig. 4), no expression of 11β-OHase mRNA could be detected in the Y1-E2, Y1-E10, Y1-E12, or Y1-E16 cell lines, even upon prolonged exposure of the blot to film. In the Y1-E15 cell line, the only Y1-E cell line which constitutively expresses 11β-OHase, 11β-OHase mRNA was stimulated cholesterol mobilization and intracellular transport.

### Table II

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ApoE mRNA</th>
<th>SCC mRNA</th>
<th>Steroid Basal +Bt2cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1-neo</td>
<td>&lt;0.1</td>
<td>100</td>
<td>pg/µg</td>
</tr>
<tr>
<td>Y1-E15</td>
<td>0.8</td>
<td>61</td>
<td>1.15</td>
</tr>
<tr>
<td>Y1-E2</td>
<td>2.9</td>
<td>19</td>
<td>0.11</td>
</tr>
<tr>
<td>Y1-E10</td>
<td>63.4</td>
<td>16</td>
<td>0.06</td>
</tr>
<tr>
<td>Y1-E12</td>
<td>120.0</td>
<td>16</td>
<td>0.19</td>
</tr>
<tr>
<td>Y1-E16</td>
<td>57.2</td>
<td>14</td>
<td>0.18</td>
</tr>
<tr>
<td>Y1-E1</td>
<td>17.2</td>
<td>9</td>
<td>0.09</td>
</tr>
</tbody>
</table>

**FIG. 2. Northern blot analysis of RNA from Y1 and Y1-E cell lines.** Top panel, blot was probed with a mouse SCC cDNA probe; the position of the approximately 2.2-kb band is indicated. Middle panel, the same blot was stripped and reprobed with a mouse 11β-OHase cDNA probe as indicated and quantified by counting excised dots by scintillation spectrometry (see Table II). Background, as indicated by hybridization to chicken liver RNA, was subtracted from each sample. Hybridization to GAPDH was used to normalize samples for variations in RNA loading. Lanes: 1, Y1; 2, Y1-neo; 3, Y1-E1; 4, Y1-E2; 5, Y1-E10; 6, Y1-E12; 7, Y1-E15; 8, Y1-E16.

**FIG. 3. Dot blot analysis of Y1, Y1-neo, and Y1-E cell lines.** Micrograms of total RNA blotted is indicated on the left side of the figure. The blot was probed with either a mouse SCC cDNA probe or a rat GAPDH cDNA probe as indicated and quantified by counting excised dots by scintillation spectrometry (see Table II). Background, as indicated by hybridization to chicken liver RNA, was subtracted from each sample. Hybridization to GAPDH was used to normalize samples for variations in RNA loading. Lanes: 1, Y1; 2, Y1-neo; 3, Y1-E1; 4, Y1-E2; 5, Y1-E10; 6, Y1-E12; 7, Y1-E15; 8, Y1-E16; 9, chicken liver RNA.

in steroid production in the individual Y1-E cell lines. Comparison of the -fold reductions of SCC mRNA and basal steroid synthesis (Table II) suggests that reduced constitutive expression of SCC can account for the majority of suppression in basal steroidogenesis we observe. For example, expression of SCC mRNA is reduced 11-fold in Y1-E1 cells, whereas basal steroidogenesis is reduced 12-fold. Likewise, SCC mRNA and basal steroidogenesis are both reduced approximately 6-fold in the Y1-E12 and Y1-E16 cell lines. In contrast to basal steroidogenesis, cAMP-stimulated steroidogenesis in the Y1-E cell lines must be inhibited at other points in addition to reduced SCC expression. For example, in the Y1-neo cell line, steroid production increases 21-fold upon cAMP stimulation, whereas in most of the Y1-E cell lines, steroid production is increased only 1.5- to 3-fold (Table II). Thus, with the exception of the Y1-E15 cell line, cAMP-stimulated steroid production is reduced 75- to 160-fold in the Y1-E cell lines, whereas SCC mRNA expression is reduced only 5- to 7-fold. These results suggest that a cAMP-stimulated component of steroidogenesis independent of SCC expression is inhibited in the Y1-E cell lines. As discussed above, one possibility is that the Y1-E cell lines are defective in cAMP-stimulated cholesterol mobilization and intracellular transport.

To determine if the constitutive expression of other steroid hydroxylase genes is suppressed in the Y1-E cell lines, we examined the expression of 11β-OHase mRNA by Northern blot analysis (Fig. 2). 11β-OHase is required for production of the final steroid product of Y1 cells, 11β, 20α-dihydropregesterone. In the absence of 11β-OHase, Y1 cells produce 20α-dihydropregesterone, also detectable in the fluorogenic steroid assay used in Table I. As seen in Fig. 2, although 11β-OHase mRNA is easily detectable in Y1-neo cells, expression is reduced to below detectable levels in all but one Y1-E cell line. In this cell line, Y1-E15, expression of 11β-OHase is reduced approximately 2-fold. The Y1-E15 cell line produces the lowest level of apoE mRNA of the clones we have examined and is the least inhibited in steroidogenesis (Table II).

In Y1 cells maintenance of basal steroidogenesis is probably dependent, at least in part, on the relatively high ACTH levels found in horse serum (21). Expression of SCC and 11β-OHase mRNA above constitutive levels can be induced by incubating cells for 12-48 h in cAMP or an activator of adenylyl cyclase such as forskolin. To determine if cAMP induction of SCC and 11β-OHase mRNA is altered in the Y1-E cell lines, we incubated cells in Bt2cAMP for 24 h and analyzed mRNA expression by Northern blot analysis. As seen in Fig. 4, SCC mRNA is induced 2- to 3-fold in Y1 and Y1-neo cells and in all the Y1-E cell lines examined. The modest increase in SCC mRNA in the Y1-E cell lines corresponds to the 2- to 3-fold stimulation of steroidogenesis observed in response to cAMP (see Table III). These results demonstrate that although constitutive expression of SCC mRNA is suppressed in the Y1-E cell lines, SCC can respond to cAMP with a fold induction comparable with that observed in Y1 or Y1-neo cells. Nonetheless, even after induction with cAMP, expression of SCC in the Y1-E cell lines is still greatly suppressed compared with the control cell lines. When cAMP stimulation of 11β-OHase mRNA was examined (Fig. 4), no expression of 11β-OHase mRNA could be detected in the Y1-E2, Y1-E10, Y1-E12, or Y1-E16 cell lines, even upon prolonged exposure of the blot to film.
Expression of ApoE Inhibits cAMP Induction of Non-steroidogenic Genes—To determine if cAMP-mediated regulation of non-steroidogenic genes is also altered in the Y1-E cell lines, we examined cAMP-induced expression of ornithine decarboxylase mRNA, a member of the immediate-early family of growth-regulated genes. Ornithine decarboxylase activity is regulated in Y1 cells in response to ACTH or cAMP stimulation (22). As seen in Fig. 5, in Y1 cells, ornithine decarboxylase mRNA is induced approximately 3- to 4-fold following a 9-h incubation with Bt2cAMP. In contrast, no induction of ornithine decarboxylase was observed in either the E12 or E16 cell lines under the same conditions. These results suggest that in addition to suppressing expression of genes required for steroidogenesis, expression of apoE in Y1 cells may also suppress cAMP-mediated regulation of some non-steroidogenic genes.

CAMP-dependent Protein Kinase Activity Is Unaffected by ApoE Expression—Studies utilizing cAMP-dependent protein kinase defective subcones of Y1 cells have demonstrated that constitutive expression of SCC is at least partially dependent upon a functional cAMP-dependent protein kinase, whereas cAMP-dependent protein kinase appears to be absolutely required for expression of the 11β-OHase gene (11). Additional defects in these kinase-defective cell lines include lack of morphologic changes in response to ACTH or cAMP (23) and a deficiency in ACTH or cAMP induction of ornithine decarboxylase activity (22). Since all known effects of cAMP occur via cAMP-dependent protein kinase (24), the inhibition in cAMP-mediated gene expression we observe in the Y1-E cells could be accounted for by a deficiency in the activity of this enzyme. To determine if this is the case, we measured protein kinase activity in Y1-E cell lines following activation by cAMP both in vitro and in vivo. As seen in Fig. 6, the protein kinase activities of the Y1, Y1-neo, Y1-E12, and Y1-E16 cell lines show essentially the same response to increasing concentrations of cAMP when assayed in vitro. This demonstrates that lack of cAMP-mediated gene expression in these cell lines is not due to decreased expression or activity of the kinase catalytic subunit. However, overexpression of the regulatory subunit, or a mutation resulting in increased affinity of the regulatory subunit for the catalytic subunit, would abolish or diminish cAMP-dependent kinase activity in vivo but might not be detectable under in vitro assay conditions. Therefore, to determine if the kinase activity measured in vitro represents kinase which can be activated in vivo, we measured the activity ratio of cAMP-dependent kinase activity with and without prior treatment of cells with Bt2cAMP in vivo. As seen in Table III, the activity ratio of cAMP-dependent protein kinase was similar in the five cell lines examined under both basal conditions and following activation of the kinase by prior treatment with cAMP in vivo. Thus we conclude that the inhibition of cAMP-mediated gene expression in the Y1-E cells lines is not due to a defect in cAMP-dependent protein kinase activity but presumably occurs at some point distal to activation of this kinase.

DISCUSSION

Adrenocortical cells in vivo, and cultured adrenal cells in vitro, utilize cholesterol for steroid hormone synthesis. In vivo...
TABLE III  
cAMP-dependent protein kinase activity ratios

The protein kinase activity ratio represents enzyme activity in vitro in the absence of cAMP divided by enzyme activity in the presence of 10^{-5} M cAMP. Measurements were done either on unstimulated cells (basal) or on cells stimulated in vitro by incubation for 60 min in complete medium containing 2 mM Bt2cAMP. Values represent the average of two or more experiments.

<table>
<thead>
<tr>
<th>Activity -cAMP/+cAMP</th>
<th>Y1</th>
<th>Y1-neo</th>
<th>Y1-E1</th>
<th>Y1-E12</th>
<th>Y1-E16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1</td>
<td>0.04</td>
<td>0.05</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Y1-neo</td>
<td>0.53</td>
<td>0.53</td>
<td>0.53</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td>Y1-E1</td>
<td>0.31</td>
<td>0.31</td>
<td>0.31</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Y1-E12</td>
<td>0.46</td>
<td>0.46</td>
<td>0.46</td>
<td>0.46</td>
<td>0.46</td>
</tr>
<tr>
<td>Y1-E16</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

studies suggest a direct correlation between rat adrenal apoE expression and total cell cholesterol and an inverse relationship between apoE expression and adrenal steroidogenesis (4). To investigate the function of apoE in functionally different cells, we have created a series of Y1 clonal cell lines which stably express a transfected human apoE gene. We have reported previously that expression of apoE in Y1 cells dramatically suppresses basal and ACTH or cAMP-stimulated steroidogenesis (9). To investigate the mechanism of this suppression, we have focused on cAMP-regulated events required for acute and chronic regulation of adrenal steroidogenesis. We report here that suppression of steroidogenesis in the Y1-E cells results, at least in part, from inhibition of cAMP-regulated gene expression. In addition, inhibition of acute responses to cAMP may indicate that intracellular cholesterol trafficking is also altered in the Y1-E cell lines.

Adrenal steroidogenesis is regulated both acutely and chronically by the pituitary hormone ACTH. Extensive evidence suggests that most, but possibly not all, of the actions of ACTH are mediated via the second messenger, cAMP (5). The acute response of adrenal cells to ACTH or cAMP includes distinct morphologic changes, cholesteryl ester hydrolysis, and increased transport of free cholesterol to the mitochondria, the site of steroid synthesis. In Y1-neo cells this results in a 4-fold increase in steroid production after 60 min of incubation with cAMP, whereas no acute increase in steroid production is detectable in the Y1-E cell lines (Table I). Data described in this paper demonstrates that Y1-E cells are also defective in the morphologic response to ACTH or cAMP (Fig. 1). These cytoskeletal changes are thought to be involved in mobilization of cholesterol from storage sites to the mitochondria (19, 20). Additionally, we have reported previously that cholesterol esterification is sustained in the Y1-E cell lines in the presence of cAMP or ACTH, conditions which suppress esterification 5-fold in the Y1 and Y1-neo cell lines (9). This result is consistent with a block in the transport of free cholesterol to the mitochondrion for steroidogenesis, a process which normally reduces the free cholesterol concentration available for esterification. Taken together, these results suggest that impaired cholesterol mobilization and/or transport may contribute to the suppression of steroidogenesis in the Y1-E cell lines.

A second action of ACTH on steroidogenesis is the long term maintenance of optimal levels of enzymes and other proteins needed for steroid synthesis. In Y1 cells these include the mitochondrial P450-steroid hydroxylases, SCC, and 11β-OHase. Our data demonstrates that the constitutive expression of SCC and 11β-OHase mRNA is severely impaired in the Y1-E cell lines. The -fold reduction in SCC mRNA corresponds to the -fold reduction in basal steroidogenesis in the individual cell lines. Thus, suppression of SCC mRNA is likely to account for reduced basal steroidogenesis in the Y1-E cells. On the other hand, reduced SCC expression alone cannot account for the 75- to 160-fold difference in cAMP-stimulated steroidogenesis in the Y1-E cell lines compared with the Y1-neo cell line. This suggests that some cAMP-stimulated component of steroidogenesis distinct from SCC expression is also impaired in the Y1-E cell lines. The absence of an acute effect of cAMP on steroidogenesis supports this hypothesis. One possibility, as suggested above, is that the Y1-E cell lines are defective in some aspect of cholesterol mobilization or transport. ACTH-regulated cholesterol transport proteins, such as SCP2, appear to be required for transport of free cholesterol to the mitochondria and from the outer to inner mitochondrial membrane (25-27). Like the steroid hydroxylase genes, activation of SCP2 requires long term (24-48 h) administration of ACTH or cAMP (8, 25).

Considering the alterations in cAMP regulation described in this paper, it is possible that regulation of a cholesterol transport protein(s) is also defective in the Y1-E cells, although this remains to be examined.

Expression of 11β-OHase is also dramatically reduced in the Y1-E cell lines. The only cell line in which we were able to detect 11β-OHase mRNA was Y1-E15, the cell line with the lowest level of apoE expression. Studies from cAMP-dependent protein kinase defective Y1 cells demonstrate that constitutive expression of SCC mRNA is regulated in both a cAMP-dependent and cAMP-independent fashion, whereas constitutive expression of 11β-OHase is absolutely dependent on cAMP (11). Thus, a block in cAMP-regulated gene expression would be expected to diminish SCC mRNA levels and essentially block expression of 11β-OHase, a situation identical to what we observe in the Y1-E cell lines. In addition to maintaining constitutive expression of steroid hydroxylases genes, ACTH or cAMP induces expression of these enzymes above constitutive levels in Y1 cells. Our data demonstrate that the -fold induction of SCC mRNA by cAMP is similar in the Y1, Y1-neo, and Y1-E cell lines. However, the absolute quantity of induced SCC mRNA is much lower in the Y1-E cell lines compared with the Y1 or Y1-neo cell lines. One explanation for these results is that different branches of the cAMP signaling pathway are responsible for constitutive versus inducible expression of SCC mRNA. Analysis of the SCC promoter suggests that distinct DNA elements may be responsible for constitutive and cAMP-inducible expression (28, 29). The similar -fold induction of SCC mRNA in the Y1, Y1-neo, and Y1-E cell lines is in contrast to the absence of cAMP induction of the acute responses noted above and of ornithine decarboxylase mRNA induction in the Y1-E cell lines. This suggests that apoE expression in Y1 cells may result in selective suppression of some, but not all, cAMP-inducible responses. Since cAMP-dependent protein kinase activation is apparently normal in the Y1-E cell lines, this selective suppression may reflect inhibition of a specific branch of the signal transduction pathway distal to kinase activation.

The manner in which cAMP responsiveness is altered in the Y1-E cell lines is not known. However, since both acute and chronic responses are blocked in the steroidogenic pathway, as well as a response unrelated to steroidogenesis (ornithine decarboxylase induction), it seems unlikely that apoE acts directly on these multiple endpoints. Rather, it seems more likely that apoE directly or indirectly alters the protein kinase pathway at a site distal to the kinase itself but still common to the various endpoints that were suppressed. cAMP regulation of SCC, 11β-OHase, and ornithine decarboxylase gene expression occurs through defined response elements in
the promoters of these genes (28–31). Thus, alteration of a trans-acting factor common to the regulation of these promoters could account for the inhibition of cAMP-regulated gene expression that we observe. This trans-acting factor is probably not the cAMP response element binding protein (CREB), since although the 11β-OHase and ornithine decarboxylase promoters have a canonical cAMP response element, the SCC promoter does not.

Both cAMP-dependent protein kinase and protein kinase C signal transduction pathways have been shown to be important in regulating steroidogenesis and may have antagonistic functions (32–34). In this regard, Ilvesmaki et al. (33) have reported in human adrenal cells, and Moore et al. (34) have reported in Y1 cells, that treatment with 12-O-tetradecanoylphorbol-13-acetate suppresses SCC expression and inhibits induction of SCC mRNA by cAMP. Thus it is possible that apoE expression alters phospholipid metabolism in the Y1-E cells in a way that influences the protein kinase C pathway or another signal transduction pathway which impinges on the cAMP-dependent protein kinase transduction pathway. Although apoE is generally considered to be a typical secretory protein, a recent immunochemical study of rat liver has reported in Y1 cells, that treatment with 12-O-tetradecanoylphorbol-13-acetate suppresses SCC expression and inhibits induction of SCC mRNA by cAMP. Thus it is possible that apoE expression alters phospholipid metabolism in the Y1-E cells in a way that influences the protein kinase C pathway or another signal transduction pathway which impinges on the cAMP-dependent protein kinase transduction pathway. Although apoE is generally considered to be a typical secretory protein, a recent immunochemical study of rat liver identified apoE in hepatocyte peroxisomes and in other cytoplasmic locations in addition to the expected secretory and endocytic pathways (35). Thus, apoE may have access to intracellular components of signal transduction pathways. In this regard, two other reported functions of apoE are also suggestive of alterations in signal transduction. In ovarian theca/interstitial cells, exogenous apoE inhibits induction of androgen synthesis by luteinizing hormone (36). ApoE also inhibits mitogen-stimulated lymphocyte activation (37); in both cases, apoE must be present prior to the hormonal or mitogenic signal to block the response. These findings as well as the results of the present study suggest that apoE may have effects on signal transduction pathways independent of its role in systemic cholesterol transport. Such actions could have diverse and significant effects on cellular metabolism and perhaps explain why apoE is expressed in a wide variety of tissues and cell types.

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REFERENCES


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