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The Interrelationship of Polycyclic Hydrocarbon Metabolism and Steroidogenesis in Primary Cultures of Bovine Adrenal Cortical Cells

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SUMMARY

The interrelationship between adrenal steroidogenesis and polycyclic aromatic hydrocarbon metabolism has been examined in cultured bovine adrenal cortical (BAC) cells. Adrenocorticotropin (ACTH) selectively induced steroidogenic cytochrome P-450-dependent enzyme activities from BAC cell cultures. In the presence of 10^{-7} M ACTH, steroid production requiring 17α-hydroxylation (cortisol + androgens) was increased 5-fold over the formation of 17-deoxysteroids (corticosterone). The effect of 10 μM benz[*a*]anthracene on steroidogenesis was characterized by suppression of both steroid 17α-hydroxylation (90%) and total steroidogenesis (50%), with a concomitant rise in 17-deoxysteroid formation. The order of stimulation of steroidogenic enzyme activities by ACTH (17α-hydroxylase > side chain cleavage > 21-hydroxylase) paralleled the order of suppression by benz[*a*]anthracene. BAC cell cultures incubated with Su-10603, a specific 17α-hydroxylase inhibitor, exhibited similar changes in the pattern of steroidogenesis, as did benz[*a*]anthracene-treated cells, suggesting that benz[*a*]anthracene also inhibits steroidogenesis as an inhibitor of 17α-hydroxylase. In addition, benz[*a*]anthracene induced benzo[*a*]pyrene metabolism 4- to 6-fold over control levels in these cells. The profile of benzo[*a*]pyrene metabolites revealed predominantly water-soluble products (nonhydrolyzable > sulfates > glucuronides), 9,10-monooxygenation products, and 3-phenol. ACTH (10^{-7} M) and 0.5 mM cyclic AMP each decreased benzo[*a*]pyrene metabolism by more than 50%. Both benz[*a*]anthracene-induced and uninduced benzo[*a*]-pyrene metabolism were equally reduced in response to ACTH and cyclic AMP. In the presence of 0.2 mM aminoglutethimide, which completely inhibited steroidogenesis, ACTH decreased benz[*a*]anthracene induction of benzo[*a*]pyrene metabolism to the same extent as ACTH treatment alone. It is concluded that the suppression of benzo[*a*]-pyrene metabolism by ACTH is mediated by cyclic AMP and does not involve steroids generated in response to ACTH. These studies demonstrate that cytochrome P-450 isozymes involved in steroidogenesis and polycyclic aromatic hydrocarbon metabolism are regulated, in opposing directions, by ACTH.

INTRODUCTION

Cholesterol is metabolized to various steroid products in the adrenal cortex by a sequence of mixed-function oxidase reactions dependent on distinct and selective forms of cytochrome P-450 (1). In addition to steroid hydroxylation, the adrenal cortex exhibits substantial activity for xenobiotic metabolism, which is also catalyzed by cytochrome P-450 (2–5). Cholesterol side chain cleavage (P-450$_{scc}$) and steroid 11β-hydroxylase (P-450$_{11\beta}$) are located in the inner mitochondrial membrane. Both enzymes require adrenodoxin and adrenodoxin reductase to mediate electron transfer from NADPH (6). Steroid 21-hydroxylase and 17α-hydroxylase, and also cytochrome P-450 enzymes which catalyze xenobiotic metabolism, are located in the endoplasmic reticulum. These enzymes require a common NADPH-cytochrome P-450 reductase to mediate transfer from NADPH (7). In addition, a third microsomal activity, C-17,20 lyase, which catalyzes the formation of androgens, also requires cytochrome P-450 and P-450 reductase (8). The purification of unique P-450 cytochromes from porcine testis and guinea pig adrenal microsomes, which exhibit both 17α-hydroxylase and C-17,20 lyase activities, has been reported (8, 9).

The total steroid production is determined by the activity of cholesterol side chain cleavage, which is dependent both on the level of cytochrome P-450$_{scc}$ and on the availability of cholesterol to this enzyme in the mitochondria (10, 11). Cholesterol is supplied to the adrenal, *in vivo*, by receptor-mediated uptake of esterified cholesterol and subsequently stored as lipid droplets

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(12). Hydrolysis of cholesterol esters and transfer of cholesterol to mitochondrial P-450$_{scc}$ are both dependent on ACTH[1] (13). The relative levels of the various steroid hydroxylases and also 17,20 lyase determine the final secretory products in the adrenal cortex. The levels of several of these enzymes have been demonstrated to be elevated by the action of ACTH on the adrenal cortex, and all activities fall drastically over several days following hypophysectomy (14). In BAC cell cultures, decreases in specific enzyme activities result in dramatic changes in steroid distribution. P-450$_{11\beta}$, for example, has been demonstrated to be remarkably sensitive to lipid peroxidation (15) or to the elevation of androgens (16). Subsequent decline in P-450$_{11\beta}$ activity results in an accumulation of 11-deoxysteroids which is not reversed by the addition of ACTH.

The synthesis of steroids and control of steroidogenesis have been studied extensively in primary cultured BAC monolayers (10, 15–18). BAC cell cultures proliferate in the presence of fibroblast growth factor and will increase production of steroids (primarily cortisol and corticosterone) when challenged with ACTH (19). Because of the ability to study hormonal regulation of cytochrome P-450, both at the enzymatic and genomic level, these cells offer an ideal model to study mechanisms involved in steroidogenesis *in vitro*.

The rate of PAH metabolism in rat adrenals is approximately one-half the activity in uninduced rat liver (2, 20), whereas in guinea pig the rate of metabolism of PAH in the two organs is approximately the same (3, 4). In addition, DMBA metabolism reportedly initiates necrosis of the two inner zones of the rat adrenal cortex (21). However, the control of xenobiotic metabolism in the adrenal cortex has received little attention. The metabolism of BP in guinea pig and rat adrenal microsomes has been demonstrated to be regulated independently from steroid hydroxylases by several physiological variables and presumably involves different forms of cytochrome P-450 (2, 4, 5). Adrenal BP metabolism in hypophysectomized rats reportedly declines and can be induced to control levels upon administration of ACTH (2). The action of several pituitary hormones also modifies the induction of hepatic cytochrome P-450 by xenobiotics (22) and the metabolism of PAH in the testis (23). We report here that BAC cultures exhibit significant BP metabolism. The interplay between a pituitary hormone (ACTH) and PAH on steroidogenesis in the adrenal is examined. We present evidence for differential regulation of steroidogenesis and hydrocarbon metabolism in these cultured adrenal cells.

MATERIALS AND METHODS

Chemicals. BP and BA (Gold Label) were purchased from Aldrich Chemical Company (Milwaukee, Wisc.) [G-^{3}H] (65 Ci/mmole) and ACS were purchased from Amersham Radiochemicals (Arlington Heights, Ill.). [^{3}H]BP was purified by reverse-phase HPLC less than 24 hr prior to use and stored at −20° in the dark. Aminoglutethimide (Elipten phosphate) and 7-chloro-3,4-dihydro-2- (3-pyridyl)-naphthalene-1(2H)-one (Su-10603) were gifts from Ciba Pharmaceuticals, Inc. (Summit, N. J.). Methanol, water, and methylene chloride for HPLC analyses were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.). All other solvents were of reagent grade or better. Radioactive peaks eluting from HPLC were quantitated in Biofluor scintillation cocktail (New England Nuclear Corporation, Boston Mass.). All other chemicals, unless noted otherwise, were purchased from Sigma Chemical Company (St. Louis, Mo.).

Preparation of adrenal cell culture. BAC monolayers were prepared according to the method of Gospodarowicz *et al.* (19). Cells were maintained in 3.5-cm DIA plastic dishes (Costar, Cambridge, Mass.) or 100-mm DIA dishes (Falcon, Cockeysville, Md.) with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Ham's F-12 medium (Grand Island Biological Company, GIBCO, Grand Island, N. Y.), supplemented with minimal essential medium amino acids and vitamins (GIBCO) and 10% fetal bovine serum (KC Biologicals, Inc., Lenexa, Kan.). Penicillin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (2.5 μg/ml) (Fungizone, GIBCO) were added to retard bacterial and fungal growth in short-term cultures. Where noted, a mixture of antioxidants was added (1 μM α-tocopherol, 100 μM ascorbic acid, and 50 nM selenite) to extend culture life-span (24). Bovine brain fibroblast growth factor was a generous gift from Dr. Denis Gospodarowicz (University of California, San Francisco, Calif.).

Bovine adrenal cell suspensions were aseptically prepared from strips of cortex (~0.5 mm thick) obtained from demedullated glands, of which the larger pieces were minced with a scalpel. The tissue fragments were washed several times and suspended in 20–30 ml of fresh medium containing collagenase (2.5 mg/ml) (Sigma type IA) plus DNAse 1 (0.1 mg/ml) (Sigma Type III, bovine pancreas) and incubated at 37° for 2 hr. Cells were then dispersed with a 10-ml plastic serological pipette, filtered through sterile cheesecloth, and washed several times by centrifugation at 200 × *g* for 20 min with fresh medium to remove collagenase. Adrenal cells were plated at a cellular density of 2 to 3 × 10^5 cells/cm^2 and maintained at 37° in a humidified atmosphere of 95% air/5% CO_2. Medium was replaced every 24 hr with fresh medium containing 10% fetal bovine serum and fibroblast growth factor (100 ng/ml) until confluency (nondividing monolayers) was reached. Typically, under these conditions, adrenal cells reached confluency within 4–6 days after plating. All cells received fresh medium + 10% fetal bovine serum, but without fibroblast growth factor, 24 hr prior to an experiment. Cell number from individual plates was quantitated from suspensions prepared by brief trypsinization of cultured cells (3 min with 0.1% trypsin at 37°) and then counted using a hemocytometer (American Optical, Buffalo, N. Y.).

Steroids secreted by BAC cell cultures were quantitated by HPLC. Steroids in the medium were extracted with methylene chloride at a ratio of 9:1 methylene chloride to medium. Cortisone, which is not produced by cultured BAC cells, was included as a standard before extraction to correct for recovery. The methylene chloride phase was separated from the aqueous phase, evaporated to dryness under N_2, and resuspended in a minimal volume of 100% methanol.

Subcellular fractionation. BAC cell homogenates were prepared from monolayers by scraping cells from dishes, incubating cells in hypotonic buffer (10 mM Tris, 10 mM KCl, and 0.5 mM EDTA) at 4° and then homogenized. The mitochondrial (10,000 × *g*) supernatant was obtained as described elsewhere (11). Enzyme activities associated with this fraction were measured by utilizing the NADPH-generating system previously reported (20), with 75 μM progesterone as substrate. Reactions were quenched after 20 min by the addition of methylene chloride and prepared for HPLC as described above. Protein concentrations were determined from trichloroacetic acid precipitates by the method of Lowry *et al* (25), with bovine serum albumin as the standard.

BP metabolism. BP metabolism was measured in BAC cell cultures

[1] The abbreviations used are: ACTH, adrenocorticotropin; PAH, polycyclic aromatic hydrocarbon(s); BAC, bovine adrenal cortical; DMBA, 7,12-dimethylbenz[*a*]anthracene; BP, benzo[*a*]pyrene; BA, benz[*a*]anthracene; HPLC, high-pressure liquid chromatography; dibutyryl cyclic AMP, N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate.

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with 5 μM [^{3}H]BP (approximately 0.5 mCi/mmole) prepared in fresh medium + 10% fetal bovine serum. BP was added to plates with cells and control plates without cells (~2.5 μCi/well) and incubated at 37°. Total oxidation of BP was measured after 4 hr as previously described (26), and radioactivity was determined in the aqueous phase by liquid scintillation counting. To identify and quantitate BP metabolites by HPLC, aliquots of medium from adrenal cell incubations were extracted with 3 volumes of ethylacetate/acetone (2:1) containing 0.15 mM dithiothreitol, and the organic phase was evaporated under N_2. Adrenal cultures were induced with BA (10 μM) or BA and ACTH (10 μM and 10^{-7} M, respectively) for 24 hr. After extensive washing to remove BA, medium containing 5 μM [^{3}H]BP (~25 μCi/dish) was added to the cells and incubated for 4 hr. In order to fractionate conjugated metabolites from free metabolites, medium from cells incubated with [^{3}H]BP was treated with β-glucuronidase or aryl sulfatase according to the method of Burke *et al.* (27). Medium was mixed with acetate buffer (0.25 M final concentration, pH 5.0) containing either β-glucuronidase (2×10^5 units/ml) (Sigma Type B-10 from bovine liver) or aryl sulfatase (10 units/ml) (Sigma Type V from limpets) plus 20 mM D-saccharic acid 1,4-lactone to inhibit contaminating β-glucuronidase. Medium to be subjected to enzyme hydrolysis was then incubated at 37° for 5 hr, and the reaction was quenched with ethylacetate/acetone as described above. Ascorbic acid (1.0 mg/ml) was added during each incubation to reduce oxidation of phenols to quinones. Metabolite profiles from enzyme-treated medium were compared with those from control medium, which was medium from [^{3}H]BP-treated cells subjected to incubation for 5 hr in equivalent buffer but without enzyme addition.

HPLC analysis. HPLC analyses were carried out as described previously (26). Steroids were resolved on an Altex/Beckman Ultrasphere Octyl column (0.46 × 25 cm) at 35° and a flow rate of 0.8 ml/min, using a methanol/water gradient. Quantitation of Δ-4,3-ketosteroids was performed by comparing UV-absorbing peaks at 254 nm (0.05 AUFS) from each extraction to known standard injections run concurrently with each analysis. BP and its primary metabolites were separated on an Altex/Beckman Ultrasphere ODS column (0.46 × 25 cm), also using a methanol/water gradient. BP metabolites were eluted at a flow rate of 1.0 ml/min. UV absorbance was monitored at 254 nm, 0.02 AUFS, and fluorescence was detected with 248-nm excitation and >389 nm emission wavelengths. In BAC incubations with [^{3}H]BP, UV and fluorescent peaks were confirmed to be BP metabolites by measuring radioactivity, eluting with each peak, with an Anspec AN-700 radioactivity LC detector. In addition, metabolites were identified by comparing metabolite retention times with those of authentic standards or metabolites from control microsomal incubations obtained as described previously (26).

RESULTS

Characterization of steroidogenesis in BAC cell culture. When primary monolayers of BAC cells were incubated with 10^{-7} M ACTH for 24 hr, a 4-fold stimulation of total steroidogenesis over basal levels was observed (Fig. 1A). The stimulation of steroidogenesis was half-maximal with 10^{-11} M ACTH and maximal with 10^{-8} M ACTH. HPLC analysis of steroids extracted from the medium of ACTH-stimulated BAC cell cultures revealed three major products, cortisol (69% of total steroids), 11β-hydroxyandrostenedione (20%), and corticosterone (11%) (Table 1). The immediate precursors to cortisol corticosterone, and 11β-hydroxyandrostenedione (11-deoxycortisol, 11-deoxycorticosterone, and androstenedione, respectively) were also detectable; however, the contribution of these precursors to total steroid in the medium was generally small (<5%). In contrast to ACTH-stimulated BAC cells, nonstimulated cells produced greater quantities of corticosterone relative to cortisol, and androgen secretion was only a small percentage of total steroid produced (Table 1). ACTH, therefore, increased the proportion of products derived from 17α-hydroxylated precursors (deoxycortisol, cortisol + androstenedione) by nearly 6-fold (Fig. 1B), whereas 21-hydroxy-17-deoxysteroid products (deoxycorticosterone + corticosterone) increased by only a factor of 1.5 (Fig. 1C). It should be noted that total steroid production by cultured BAC cells depends upon culture conditions and time of cells in culture. Although total steroid production may change (compare total steroids in Table l with those in Fig. 1), the relative proportion of individual steroid products does not.

Since deoxycorticosterone is a poor substrate for 17α-hydroxylation, the ratio of 17α-hydroxysteroids to 21-hydroxy-17-deoxysteroids (17α/21) is determined by relative rates of 17α- and 21-hydroxylation of progesterone.[2] In BAC cells, the 17α/21 ratio increased 4-fold after a 24-hr exposure to ACTH (Table 1), implying that the 17α-hydroxylation of progesterone was increased approximately 4-fold over the competing 21-hydroxylation of progesterone.

Subcellular steroidogenic activities. The microsomal metabolism of progesterone was examined in 10,000 × *g* supernatants isolated from BAC cell cultures at 0, 8, and 24 hr following ACTH administration (Table 2). In fractions isolated from unstimulated cells, no changes in the relative enzyme activities were observed. ACTH increased 17α-hydroxylation of progesterone by 10-fold while increasing the production of 21-hydroxylated products (11-deoxycortisol + 11-deoxycorticosterone) by 2-fold. The production of 11-deoxycorticosterone, from progesterone, did not change during the time course, suggesting that the increased 21-hydroxylase acitvity is likely the result of increased levels of 17α-hydroxyprogesterone, the preferred substrate for 21-hydroxylase. These changes in 17α-hydroxyprogesterone levels also prevent any conclusions concerning the effect of ACTH on C-17,20 lyase levels.

PAH effect on steroidogenesis. Because of the well-documented toxicity of DMBA in the adrenal, we initiated studies to examine the effect of BA, the generally less toxic parent hydrocarbon, on adrenal steroidogenesis in BAC cell cultures. BA, when added to the cell culture medium for 24 hr at a concentration of 5 μM or less, did not affect total basal steroidogenic activity. In the presence of 10 μM BA, however, total steroidogenesis was suppressed by 25–40% (Table 1; Fig. 2A). There were no morphological changes in cell appearence between cultures treated with or without BA, and examination of cell viability with trypan blue revealed that BA, at a concentration up to 20 μM, was not cytotoxic to the cells (data not shown).

The distribution of steroid products was, however, greatly affected by even 1.5 μM BA (Fig. 2B). The redis-

[2] The steady state for progesterone; $[PROG]_{ss} = (k_a C)/(k_c + k_d)$, assuming $k_b \gg k_a C$, where C = [cholesterol] and k_a, k_b, k_c, and k_d refer to rate constants as defined in Fig. 5. *A* (rate of 17α-hydroxysteroid formation) = $(k_c k_a C)/(k_c + k_d)$; *B* (rate of 21-hydroxy-17-hydroxysteroid formation) = $(k_d k_a C)/(k_c + k_d)$. Ratio: $A/B = k_c/k_d$ (also assumes: $k_e \gg k_c$ and $k_g \ll k_d$).

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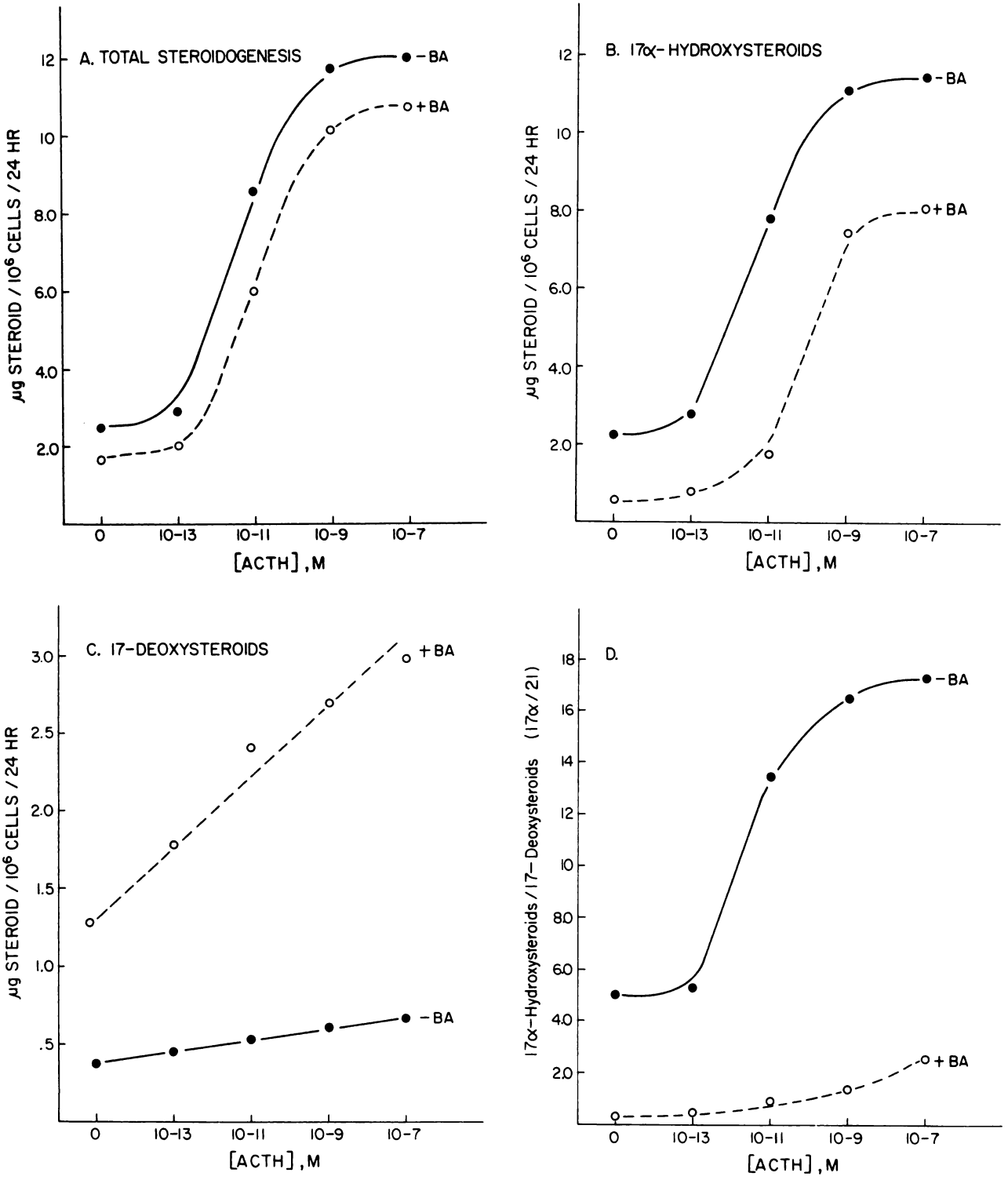

FIG. 1. *Dose-response of ACTH in stimulating steroidogenesis in the presence or absence of BA*

BAC cell cultures were incubated for 24 hr at 37° in the presence of ACTH, at the indicated concentrations, with (○) or without (●) 10 μM BA in medium containing antioxidants. Steroids were extracted from the medium and quantitated by HPLC as outlined under Materials and Methods. Results are expressed as the average micrograms of steroid/10^6 cells/24 hr of two experiments. A, Total steroidogenesis; B, total 17α-hydroxysteroids; C, total 17-deoxysteroids; D, 17α-hydroxysteroid/21-hydroxy-17-deoxysteroid product ratio (17α/21).

tribution is the result of a dose-related decrease in the 17α/21 ratio (Fig. 2C) by BA. In addition, the formation of androstenedione was completely prevented, whereas progesterone, which was previously undetected, became observable in unstimulated (non-ACTH-treated) cells (Table 1). The absolute levels of 21-hydroxy-17-deoxysteroids (corticosterone + deoxycorticosterone), following 10 μM BA treatment, increased almost 2-fold over control cells, whereas the absolute levels of 17α-hydroxysteroids (deoxycortisol, cortisol + androstenedione) de-

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TABLE 1

Comparison of the distribution of steroid products in BAC cell cultures treated with BA or Su-10603

ACTH (10^{-7} M), BA (10 μM), and Su-10603 (0.1 μM and 10 μM) were added to BAC cell cultures in the medium as indicated under cell treatment. The cells were then incubated at 37° in an atmosphere of 95% air/5% CO_2. After 24 hr, an aliquot of medium was removed and the steroids were extracted with methylene chloride. Steroid products were resolved by HPLC (see Materials and Methods) and quantitated by comparison to known standards. Steroid amounts in each group are averages ± standard error of the mean for three experiments. The percentage of total steroid that each product represents is indicated in parentheses.

Cell treatment	Steroids in the medium					17α/21[a]
	Cortisol	Corticosterone	Androgens	Progesterone	Total	
	ng/10^6 cells/24 hr					
Control						
−ACTH	1080 ± 190 (62)	610 ± 90 (36)	70 ± 1 (4)	ND[b] (0)	1760 ± 280	1.9
+ACTH	3330 ± 120 (69)	545 ± 85 (11)	975 ± 200 (20)	ND (0)	4850 ± 490	7.9
BA						
−ACTH	80 ± 35 (6)	1010 ± 95 (79)	ND (0)	200 ± 60 (15)	1290 ± 175	0.08
+ACTH	1510 ± 440 (48)	1610 ± 300 (52)	ND (0)	ND (0)	3120 ± 565	0.9
Su-10603 (0.1 μM)						
−ACTH	175 ± 1 (11)	1325 ± 85 (84)	ND (0)	75 ± 1 (5)	1575 ± 100	0.13
+ACTH	2650 ± 360 (57)	1970 ± 125 (43)	ND (0)	ND (0)	4620 ± 485	1.3
Su-10603 (10 μM)						
−ACTH	ND (0)	1475 ± 25 (91)	50 ± 9 (3)	100 ± 25 (6)	1625 ± 275	0.03

[a] The ratios of total 17α-hydroxysteroids to 21-hydroxy-17-deoxysteroid products.
[b] ND, No steroid detected.

TABLE 2

Microsomal activities in 10,000 × g supernatants isolated from BAC cell cultures

Mitochondrial supernatants (10,000 × g) were isolated from primary BAC cell cultures with or without prior ACTH treatment at the indicated times. Products formed from progesterone (75 μM) in 20 min were analyzed by HPLC as described under Materials and Methods. Each activity represents the average of two experiments.

Products[a]	OHr[b]	8 Hr		24 Hr	
		−ACTH	+ACTH	−ACTH	+ACTH
	pmoles/mg protein/min				
17α-Hydroxysteroids	65	60	220	60	650
21-Hydroxysteroids	75	80	85	70	140
Androstenedione	10	12	25	10	75

[a] 17α-Hydroxysteroids, 11-deoxycortisol + androstenedione; 21-hydroxysteroids, 11-deoxycortisol + 11-deoxycorticosterone.
[b] OHr, cells were fractionated without prior incubation.

creased by a factor of 14. In ACTH-stimulated cells, the absolute increase in corticosterone was even larger (3-fold) in the presence of 10 μM BA, whereas the absolute level of 17α-hydroxylated products decreased by only a factor of 3. Therefore, in both ACTH-stimulated and unstimulated BAC cells, BA greatly increased the proportion of 17-deoxysteroids (corticosterone) in the medium. These results suggest a selective inhibitory effect of BA on steroid 17α-hydroxylation and cholesterol side chain cleavage activities.

Suppression of steroidogenesis by BA at various doses of ACTH. Fig. 1A and B shows that the sensitivity of total steroids and 17α-hydroxylated steroids to BA was dependent on the dosage of ACTH. In maximally stimulated cells (10^{-7} M ACTH), BA did not affect total steroidogenesis, whereas in unstimulated or slightly stimulated cells (10^{-11} M ACTH), BA suppressed total steroidogenesis by 30% (see also Fig. 2A and Table 1). This suggests some capacity of ACTH to compensate for the suppressive effects of BA. Similarly, the effect of BA on 17α-hydroxylation was greatest at low doses of ACTH. BA caused an 80% decrease in 17α-hydroxysteroid formation at ACTH concentrations below 10^{-11} M, whereas in maximally stimulated cells the reduction in 17α-hydroxysteroids was less than 30%. The magnitude of effect by BA varied between cell preparations, but the reduction in 17α-hydroxysteroids at low ACTH concentrations was always 2- to 3-fold greater than at maximal ACTH stimulation. This suppression of steroid 17α-hydroxylation by BA resulted in an increase in 17-deoxysteroid levels which was only slightly affected by the increasing ACTH concentrations (Fig. 1C). The ratio of 17-deoxysteroid production between cells incubated in the presence and absence of BA, at varying doses of ACTH, is only elevated from approximately 3 in unstimulated cells to 4.5 in maximally stimulated cells. This small increase in 17-deoxysteroid production may reflect a small stimulation in the 21-hydroxylation of progesterone by ACTH in BAC cells.

ACTH increased the 17α/21 ratio in cells by a factor of 5, irrespective of the presence of BA. Conversely, BA lowered the absolute ratio by about a factor of 10 at all

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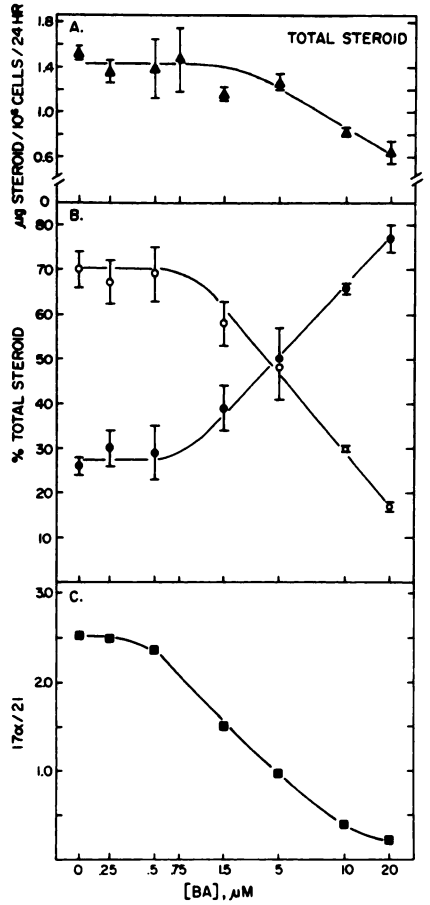

FIG. 2. *Inhibition of total steroidogenesis and 17α-hydroxylase activity by various concentrations of BA*

The dose-response of BA effects on steroidogenesis was examined in primary BAC cell cultures. BA was added to cell culture medium at the indicated concentrations and incubated with cells for 24 hr at 37°. Steroids were extracted and separated by HPLC.

A. Effect of BA on total steroidogenesis. Results are expressed as micrograms of steroid/10^6 cells/24 hr ± standard error of the mean for two incubations.

B. Effect of BA on steroid 17α-hydroxylation. Results are expressed as percentage of total steroid production for each concentration of BA ± standard error of the mean for two incubations. ○, 17α-Hydroxysteroids; ●, 17-deoxysteroids.

C. Effect of BA on the ratio of 17α-hydroxysteroids/21-hydroxy-17-deoxysteroids (17α/21).

concentrations of ACTH (compare −BA and +BA for all concentrations of ACTH, Fig. 1D). These data suggest that ACTH does not effect the suppressive action of BA but compensates for the decline in 17α-hydroxylated steroids by stimulating steroid 17α-hydroxylation in these cells. The absolute 10-fold reduction in the 17α/21 ratio indicates a 90% suppression of cellular 17α-hydroxylase activity by 10 μM BA in the presence or absence of ACTH.

Comparison of BA inhibition of 17α-hydroxylase with Su-10603. To characterize further the effect of BA in suppressing steroid 17α-hydroxylation in BAC cells, steroidogenesis in cells exposed to BA was compared with that in cells treated with Su-10603 (Table 1), a specific competitive inhibitor of microsomal 17α-hydroxylase (28). In unstimulated cells, 0.1 μM Su-10603 decreased the 17α/21 product ratio by approximately 15-fold when compared with control levels. The pattern of distribution of steroids closely resembled that of BA-treated cells. The relatively smaller accumulation of progesterone and the higher 17α/21 ratio indicate that 0.1 μM Su-10603 is approximately 2-fold less effective in suppressing steroid 17α-hydroxylation than 10 μM BA. ACTH-stimulated cells exposed to 10 μM BA or 0.1 μM Su-10603 produced similar changes in the pattern of steroid synthesis except that, at concentrations ranging from 0.1 μM to 50 μM (data not shown), Su-10603 did not affect total steroidogenesis in either ACTH-stimulated or unstimulated cells. Finally, in cells exposed to 10 μM Su-10603 or greater, the production of cortisol was no longer detectable, indicating a total inhibition of 17α-hydroxylase activity. These data confirm that Su-10603 is a more potent and specific inhibitor than BA.

Metabolism of PAH in BAC cell cultures. To establish whether BAC cells will metabolize PAH and whether this metabolism is inducible, cells were incubated at 37° in the presence or absence of BA (10 μM) for 24 hr. Control levels of BP metabolism varied from one cell preparation to another; however, in general it was in the range of 0.05–0.10 nmole of metabolites/10^6 cells/hr. The presence of BA in the 24-hr preincubation induced BP metabolism by an average of 4-fold over control (Fig. 3, 0 M ACTH). The induction of BP metabolism by BA occurred at a concentration of BA (0.75 μM; data not shown) that was well below the concentration of BA (>5 μM) which suppressed total steroidogenesis and at a concentration where a decrease in cellular 17α-hydroxylation was not detectable (Fig. 2B). BAC cells, when exposed to various doses of ACTH for 24 hr and then incubated with [^{3}H]BP for 4 hr, exhibited a marked decrease in BP metabolism as compared with metabolism without ACTH (Fig. 3, *−BA*). In addition, when ACTH and BA were co-incubated for 24 hr prior to [^{3}H]BP, the induced levels fell by more than 60% at maximal levels of ACTH stimulation as compared with BA-induced levels without ACTH (Fig. 3, *+BA*). Interestingly, the dose response of ACTH in decreasing BA-induced, BP metabolism was parallel to the dose response of ACTH in stimulating steroidogenesis. This suggests that the mechanism of action of ACTH in decreasing BP metabolism may be similar to that by which ACTH stimulates steroidogenesis.

Because of the similar effects of Su-10603 and BA on steroidogenesis, the induction of BP metabolism by Su-10603 was compared with that by BA. The results of preexposure of cells to Su-10603 (0.1 μM–10 μM) with and without BA (10 μM) are shown in Table 3. Su-10603 induced BP metabolism in a dose-dependent manner, with maximal induction reaching approximately that of 10 μM BA (~0.40 nmole/10^6 cells/hr). When incubated in the presence of BA, Su-10603 at the same doses did not affect BA induction, suggesting that the mechanism of induction of BP metabolism by both compounds is similar.

Characterization of the suppression of BP metabolism by ACTH. One explanation of the effect of ACTH on BP metabolism is that it is mediated by steroids secreted in

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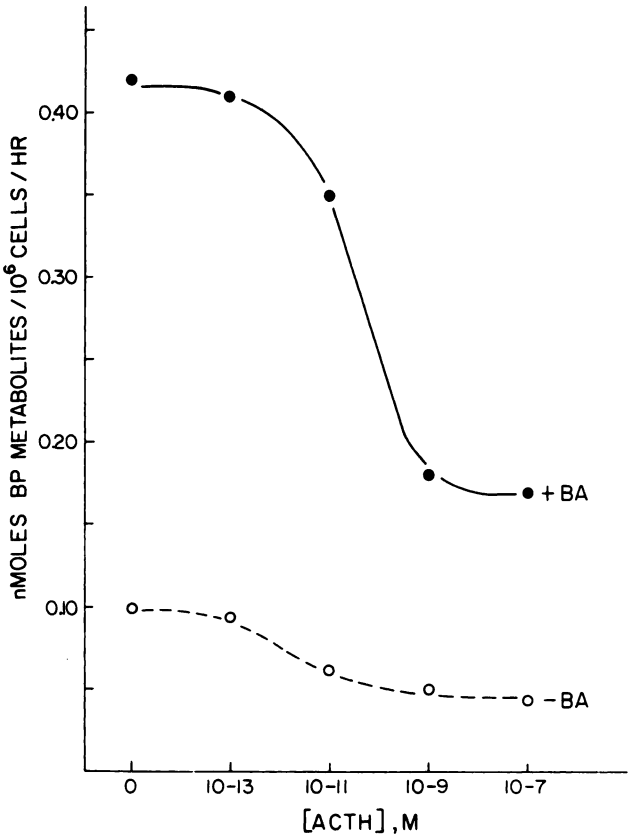

FIG. 3. *Effect of ACTH and BA on BP metabolism in BAC cell cultures*

Cultured BAC cells were incubated at 37° in the presence of ACTH, at the indicated concentrations, with (●) or without (○) 10 μM BA. After 24 hr, the cells were washed, and 5 μM [^{3}H] BP in fresh medium was added to cells at 37° for 4 hr. Oxidized BP was extracted and quantitated as described under Materials and Methods. Results are expressed as the average nanomoles of BP metabolites/10^6 cells/hr ± standard error of the mean for two experiments.

response to ACTH. To investigate this possibility, ACTH and BA were added to the medium in the presence or absence of an inhibitor of cholesterol side chain cleavage, aminoglutethimide (11), at a concentration (0.2 mM) that completely inhibited steroidogenesis in ACTH-stimulated or unstimulated BAC cells. Neither BA induction of BP metabolism in the absence of ACTH nor the decrease in BP metabolism in the presence of ACTH was affected by the simultaneous presence of 0.2 mM aminoglutethimide (Fig. 4). In addition, several of the most prominent steroids secreted by BAC cell cultures (androstenedione, 5 μM; 11β-hydroxyandrostenedione, 5 μM; corticosterone, 5 μM; cortisol, 20 μM) were individually added back to the culture medium for 24 hr in the presence of BA and aminoglutethimide. No change in BA induction of BP metabolism was observed in the presence of any of these steroids (data not shown). The results of these experiments indicate that steroids secreted by BAC cell cultures do not affect induction of BP metabolism by BA.

Finally, in order to investigate whether the effect of ACTH in decreasing BP metabolism was mediated by cyclic AMP, dibutyryl cyclic AMP was added to cells in

TABLE 3

Comparison of the induction of BP metabolism in BAC cell cultures with Su-10603 and BA

Primary cultures of bovine adrenal cells were incubated at 37° for 24 hr in the presence of varying concentrations of Su-10603 with or without 10 μM BA as indicated. After the 24-hr preincubation, the cells were extensively washed with fresh medium, and then medium containing 5 μM [^{3}H]BP was added to each plate. Metabolites of BP secreted into the medium after a 4-hr incubation were extracted and quantitated by liquid scintillation counting as described under Materials and Methods. BP metabolism is represented as average metabolite concentration ± standard error of the mean for two experiments.

Cell treatment	Su-10603 concentration	BP metabolism
		nmoles/10^6 cells/hr
Control	—	0.07 ± 0.002
BA (10 μM)	—	0.40 ± 0.03
Su-10603	10 μM	0.35 ± 0.08
	1.0 μM	0.26 ± 0.01
	0.1 μM	0.15 ± 0.02
Su-10603 + BA	10 μM	0.35 ± 0.03
	1.0 μM	0.36 ± 0.04
	0.1 μM	0.33 ± 0.04

the presence or absence of BA (Table 4). At 0.5 mM cyclic AMP, the concentration which exhibited stimulation of steroidogenesis equivalent to ~65% of a maximal dose of ACTH, the induction of BP metabolism by BA was reduced by ~50%. This is in good agreement with the decrease in BP metabolism by a maximal dose of ACTH. When cyclic AMP was administered at a concentration of 1.0 mM, there was a slight increase in steroidogenesis in the presence of BA but no difference with respect to the decrease in BA induction of BP metabolism from what was observed at 0.5 mM cyclic AMP. Interestingly, a similar decrease in basal levels of BP metabolism was observed with cyclic AMP as was observed with ACTH. These results suggest that the effect of ACTH on BP metabolism is mediated by cyclic AMP.

Profile of BP metabolites secreted by BAC cell cultures. HPLC analysis of BP metabolites, formed by BAC cultures, is shown in Tables 5 and 6. When the medium is extracted directly before hydrolysis, the majority of ^{3}H-associated metabolites (~90%) elute in the polar region of the gradient. BA increases and ACTH decreases metabolism predominantly through changes in these polar metabolites (Table 5). If the medium is first hydrolyzed non-enzymatically at pH 5.0, 20–30% of the highly polar region is released into the less polar regions of the gradient. Although most metabolites were increased by the acid treatment, the largest increases occurred with three unidentified peaks arising presumably from the acetate treatment (Table 6, UAM). Addition of aryl sulfatase resulted in a further release of a small portion of metabolites (~20% over acetate treatment alone), whereas β-glucuronidase resulted in an even smaller release of metabolites over background (10%). The largest proportion of polar products, however, remained resistant to hydrolysis.

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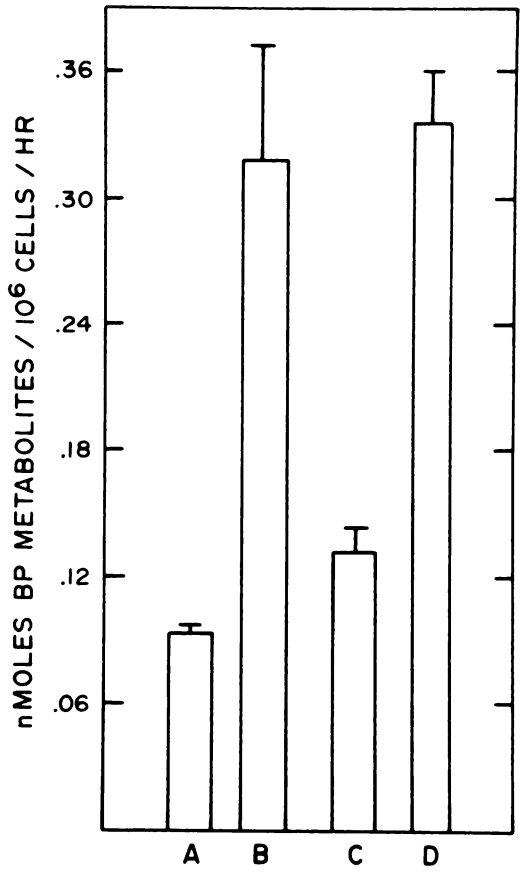

FIG. 4. *Suppression of BA induction of AHH activity by ACTH in the presence of aminoglutethimide*

Cultured BAC cells were incubated for 24 hr at 37° in medium containing: *A*, control; *B*, 10 μM BA; *C*, 10 μM BA + 0.1 μM ACTH + 0.2 mM aminoglutethimide; *D*, 10 μM BA + 0.2 mM aminoglutethimide. Cells were washed extensively, followed by a 4-hr incubation with 5 μM [^{3}H]BP. Oxidized BP was extracted and quantitated as described under Materials and Methods. Results are expressed as nanomoles of BP metabolites/10^6 cells/hr ± standard error of the mean for two experiments.

The phenols and quinones were among the most prominent identifiable BP metabolites formed in BAC cell cultures. Among the other metabolites identified by HPLC were small quantities of 9,10- and 7,8-diols. Although BA induced total metabolism by 4-fold, this induction was only observed as an increase in polar metabolites. Presumably, conjugation and secondary monooxygenation reactions have been induced to the same extent as primary monooxygenation. The levels of primary BP metabolites reflect a steady state in cells in which primary metabolism of BP is balanced by conjugation and further monooxygenation. Interestingly, the BP tetrols, derived from the carcinogenic BP 7,8-dihydrodiol-9,10-oxides which are usually not subjected to further metabolism, were not detectable in these cells.

DISCUSSION

Recent work has shown that bovine adrenal cortical cells in culture exhibit all of the steroidogenic activities found *in vivo* (10, 15, 17, 18). Figure 5 outlines the major pathways of steroidogenesis in cultured BAC cells. Confluent, primary cultures are highly responsive to ACTH,

TABLE 4

Effect of cyclic AMP on BA induction of BP metabolism in primary BAC cell cultures

ACTH (10^{-7} M) and dibutyryl cyclic AMP (0.5 and 1.0 mM) were added to the culture medium at the indicated concentrations in the presence or absence of 10 μM BA. Medium was then added to cultured BAC cells and incubated at 37°. At the end of 24 hr, the medium was extracted for total steroids and the cells were then incubated with fresh medium containing 5 μM [^{3}H]BP for an additional 4 hr. BP metabolites were extracted and quantitated by liquid scintillation counting. Steroids were quantitated by HPLC. Rate of BP metabolism and steroid production are averages ± standard error of the mean for two experiments.

Preincubation (24 hr)	BP metabolism	Total steroid
	nmoles/10^6 cells/hr	*μg/10^6 cells/24 hr*
Control	0.12 ± 0.006	2.41 ± 0.08
BA	0.45 ± 0.04	1.67 ± 0.07
ACTH		
−BA	0.08 ± 0.003	12.0 ± 0.75
+BA	0.20 ± 1	10.5 ± 0.81
Cyclic AMP (0.5 mM)		
−BA	0.08 ± 0.01	7.90 ± 0.45
+BA	0.23 ± 0.007	6.62 ± 0.04
Cyclic AMP (1.0 mM)		
−BA	0.09 ± 0.001	9.00 ± 0.63
+BA	0.21 ± 0.008	7.43 ± 0.19

producing cortisol (Pathway *I*) as the predominant steroid product and lesser amounts of androstenedione (Pathway *III*) and corticosterone (Pathway *II*). The rate of steroidogenesis in these cultured cells is comparable to that found in freshly isolated tissue (10). In this paper, we confirm the recent findings (17, 18) that the stimu-

TABLE 5

Changes in distribution of polar BP metabolites formed in BAC cell cultures

Primary BAC cell cultures were induced with BA (10 μM) or BA (10 μM) + ACTH (0.24 μM), or not induced (control), for 24 hr at 37° in medium containing antioxidants. The cells were then washed extensively and incubated for an additional 4 hr with 5 μM [^{3}H]BP (~25 μCi/dish) in fresh medium. BP metabolites from five plates were pooled and subjected to acetate hydrolysis at 37° in the presence of aryl sulfatase or β-glucuronidase, or no enzyme addition. BP metabolites were identified by HPLC and quantitated by liquid scintillation counting as described under Materials and Methods.

Cell treatment	Total polar[a]				Total metabolism[b]
	−Ac	ΔAc	ΔS	ΔG	
	pmoles/10^6 cells/hr				*pmoles/10^6 cells/hr*
Control	140	−50	−65	−55	150 ± 22
BA	530	−120	−145	−135	570 ± 55
BA + ACTH	340	−65	−90	−70	350 ± 38

[a] Total polar metabolites that elute within the first 10 min of the gradient; ΔAc, Difference between acetate-treated medium and no treatment (−Ac); ΔS, difference between aryl sulfatase treatment and acetate treatment alone; ΔG, difference between β-glucuronidase treatment and acetate treatment alone. Data represent the average from two similar experiments.

[b] Total metabolism as quantitated by dimethyl sulfoxide/hexane extraction. Values are averages ± standard error of the mean (n = 6).

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TABLE 6

Profile of BP metabolites[a] formed in primary BAC cell cultures

Conditions are identical with that described in Table 5.

Cell treatment	2°	Diols			Quinones		Phenols		UAM
		9,10	4,5	7,8	1,6	3,6	9	3	
	pmoles/10⁶ cells/hr								
Control									
−Ac[b]	ND[c]	<1	1.0	3.0	<1	1.0	1.5	4.0	ND
ΔAc	2.0	—	—	—	5.5	4.5	1.5	2.0	36
ΔS + ΔG	—	2.0	—	0.5	—	2.0	4.0	9.5	1.0
BA									
−Ac	ND	1.0	1.0	2.0	1.0	2.0	1.5	6.0	ND
ΔAc	4.0	—	—	—	6.5	5.0	5.5	6.0	36
ΔS + ΔG	5.0	4.0	1.0	3.0	2.0	1.0	6.0	5.0	20
+BA +ACTH									
−Ac	ND	<1	<1	1.5	1.0	1.5	1.0	5.5	ND
ΔAc	4.5	1.0	—	—	4.0	3.0	3.0	13	25
ΔS + ΔG	—	2.0	1.0	2.0	5.0	4.5	8.0	—	9.0

[a] Metabolite abbreviations: 2°, metabolite eluting in the region of secondary metabolites; 9,10-diol, *trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene; 7,8-diol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; 4,5-diol, *trans*-4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene; 9-phenol, 9-hydroxybenzo[*a*]pyrene; 3-phenol, 3-hydroxybenzo[*a*]pyrene; UAM, summation of three nonpolar metabolites arising from acetate hydrolysis.

[b] −Ac, Metabolite profile in medium without further treatment; ΔAc; changes in metabolite profile in medium subjected to acetate hydrolysis versus no treatment; ΔS + ΔG, summation of the differences in aryl sulfatase and β-glucuronidase treatment versus acetate treatment alone.

[c] ND, None detected.

lation of steroidogenic cytochrome P-450-dependent enzyme activities by ACTH is very selective. We also demonstrate that these enzyme activities are selectively suppressed by PAH, as represented by BA. In addition, we have established an interrelationship between ACTH and PAH in regulating cytochrome P-450 activities in these cells.

The result of selective increases and decreases in individual enzyme activities, in relation to cellular steroidogenesis, can be clarified by considering the competition for progesterone by 17α- and 21-hydroxylases (enzymes *c* and *d*, respectively; see Fig. 5). A simple evaluation of the steady state of progesterone that exists for cholesterol metabolism in either fully ACTH-stimulated or unstimulated cells indicates that the ratio of 17α-hydroxysteroids to 21-hydroxy-17-deoxysteroids (17α/21) is equivalent to the ratio of k_c/k_d, with progesterone as substrate.[2] During a 24-hr incubation in the presence of ACTH (Fig. 1; Table 1), this steady state continuously changes as the ratio of the two hydroxylases changes. Nevertheless, the 17α/21 ratio represents an average of the ratio of 17α-hydroxylation/21-hydroxylation of progesterone (*c*/*d*), weighted to the end of the 24-hr incubation where the over-all rate of steroidogenesis is highest. The 4-fold increase in the 17α/21 product ratio, produced by ACTH, is fully consistent with the ratio of enzyme activities in the postmitochondrial supernatant from ACTH-stimulated cells (Table 2). In comparison, mitochondrial cholesterol side chain cleavage activity is increased 2.5-fold in these cells after 24-hr exposure to ACTH.[3] The selective responses to ACTH described here are generally in agreement with those found for BAC cells cultured under different culture conditions. A somewhat higher induction of 17α-hydroxylase by ACTH and a lack of sensitivity of 21-hydroxylase to ACTH have been reported (17, 18). However, in the latter case, a 15-fold induction of the specific cytochrome $P\text{-}450_{21}$ (21-hydroxylase) was evident.

The effects of low levels of BA on steroidogenesis is characterized by a suppression of cortisol and androstenedione production and concomitant rises in corticosterone and progesterone. A specific 17α-hydroxylase in-

[3] M. J. DiBartolomeis and C. R. Jefcoate, submitted for publication.

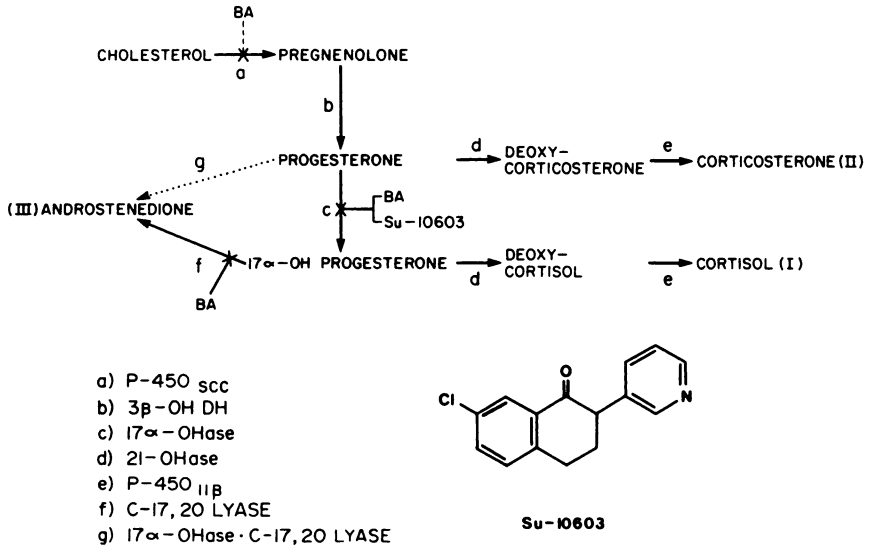

FIG. 5. *Proposed pathways for steroidogenesis in BAC cell cultures and points of inhibition by BA and Su-10603*

The primary pathways involved in conversion of cholesterol to C-21 and C-19 steroids in BAC cell cultures are presented. Enzymes are designated by small letters and defined above. Enzyme *g* denotes a proposed pathway by which androstenedione is synthesized from progesterone by one enzyme containing both 17α-hydroxylase and C-17,20 lyase activities. Points of inhibition in the sequence by BA and Su-10603 are denoted by an *X* in the *arrow* from substrate to product. The point of inhibition by BA at Step *a* is represented by a *dashed line* designating inhibition by BA at only high concentrations. The structure of the competitive 17α-hydroxylase inhibitor, Su-10603, is also shown. Enzyme abbreviations: $P\text{-}450_{scc}$, cholesterol side chain cleavage; 3β-OH DH, 3β-hydroxysteroid dehydrogenase; 17α-OHase, 17α-hydroxylase; 21-OHase, 21-hydroxylase; $P\text{-}450_{11\beta}$, 11β-hydroxylase.

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hibitor, Su-10603 (28), exhibited changes in the pattern of steroidogenesis very similar to those exhibited by BA. The data presented in Figs. 1 and 2 indicate that steroid 17α-hydroxylation in BAC cells is about 10 times more sensitive to BA (half-maximal effect at 1–3 μM) than cholesterol side chain cleavage (half-maximal effect at >20 μM). The decrease in total steroid production by BAC cells may also be the result of BA's acting by a mechanism which decreases the availability of cholesterol to P-450$_{scc}$, rather than as an inhibitor of cholesterol SCC. Although BA exerts its effects on steroidogenesis at low concentrations (1.5 μM), the suppressive action of BA is less selective on steroidogenesis than is the action of Su-10603. The changes in the 17α/21 ratio (Table 1) indicate that 0.1 μM Su-10603 inhibits 17α-hydroxylase activity by about 90% (assuming no effect on 21-hydroxylase), whereas a 10 μM concentration of this inhibitor has no effect on P-450$_{scc}$ as represented by total steroidogenesis. The total reduction of androstenedione synthesis by both compounds may partially be the result of a direct effect on C-17,20 lyase (see Fig. 5). Interestingly, at a large dose of Su-10603 (10 μM), which resulted in no detectable cortisol production, low levels (equivalent to basal levels) of androstenedione were present in the medium (Table 1). This suggests that an alternate pathway leading to androstenedione synthesis can be utilized which is catalyzed by a single enzyme with both 17α-hydroxylase and C-17,20 lyase activities. Cytochrome P-450 enzymes from testicular and adrenal microsomes (8, 9) have been isolated which exhibit both activities. The importance of such an enzyme in adrenal steroidogenesis remains to be determined.

There have been few reports which demonstrate the induction of xenobiotic metabolism in the adrenal gland. Berry *et al.* (29) demonstrated a 5-fold induction in BP metabolism by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in pregnant rats. ACTH has also been reported to increase this activity in hypophysectomized rats (2). However, in the latter study, ACTH probably increases PAH metabolism by raising microsomal cytochrome P-450 from reduced levels (~50% of control) to normal basal levels. BAC cells in culture have substantially greater levels of BP-monooxygenase activity than does fresh BAC tissue. This may reflect a selection of a minor population of BAC cells by the culturing procedure or a change in the cytochrome P-450 form responsible for this activity due to the culture conditions. The 3- to 5-fold induction of BP metabolism by BA is typical of fibroblasts (30). However, both ACTH and dibutyryl cyclic AMP, at levels which maximally stimulate steroidogenesis, decrease BA-induced and uninduced metabolism of BP in these cells. Steroids secreted by ACTH-stimulated BAC cells could possibly be inhibiting the induction of BP metabolism by BA. However, a similar decrease in this induction of BP metabolism is observed when steroid synthesis is fully inhibited by aminoglutethimide (see Fig. 4). This implies that BP metabolism occurs in cells which are responsive to ACTH treatment, i.e., an adrenal cell and not a fibroblast.

ACTH is apparently acting through cyclic AMP in decreasing PAH metabolism, since essentially identical results are obtained by both agents. In addition, it seems likely that a maximal 2-fold reduction of BP metabolism is attained with ACTH and dibutyryl cyclic AMP at 10^{-8} M and 0.5 mM concentrations, respectively. Interestingly, the doses at which ACTH stimulated steroidogenesis and decreased BA induction of BP metabolism were similar, indicating further the possibility of a common mechanism by which ACTH regulates these two distinct metabolic activities. Although dibutyryl cyclic AMP and ACTH exert effects of similar magnitude on BP metabolism in BAC cell cultures, the degree of stimulation of steroidogenesis by dibutyryl cyclic AMP at 1.0 mM is substantially less than that observed with 10^{-8} M ACTH. This may reflect a more direct mode of action by cyclic AMP on the induction of BP metabolism in comparison to that of ACTH.

Analysis of the products of BP metabolism indicates only low levels of dihydrodiols, quinones, and phenols. The production of these metabolites appears to be relatively insensitive to induction by BA, even though total metabolism is induced by 4-fold. The majority of products (>90%) are polar, suggesting that the primary product distribution reflects steady states that are insensitive to BA as a result of similar increases in both primary and secondary reaction rates. Positions 9,10 and 3 of BP appear to be favored for monooxygenation in both BA-induced and uninduced cells, whereas low epoxide hydrolase limits the formation of 9,10-dihydrodiol (hence the accumulation of 9-phenol). The nature of the extremely high levels of polar products remains to be determined. However, Burke *et al.* (27) reported a similar highly polar, nonhydrolyzable peak of BP metabolites formed in isolated hepatocytes.

Our results demonstrate that the cytochrome P-450 isozymes involved in steroidogenesis and PAH metabolism are separately controlled in BAC cells. In addition, our data provide evidence that these cytochrome P-450-dependent enzymes may be distinct, confirming previous studies in other laboratories (2–5). The order of sensitivity to stimulation of steroidogenic activity by ACTH (17α-hydroxylase > P-450$_{scc}$ > 21-hydroxylase) parallels the order of suppression by BA. BA does not seem to affect mitochondrial 11β-hydroxylase activity, as evidence by the lack of accumulation of 11-deoxysteroid products in the presence of BA. There exist several explanations for the action of BA in suppressing these steroidogenic-associated cytochrome P-450-dependent activities. Although we cannot exclude an effect of BA upon the stimulation of steroidogenic activity by ACTH, the data are fully consistent with a direct selective inhibition by BA (or a metabolite of BA) similar to that demonstrated by Su-10603. This is further supported by the evidence that Su-10603 induced BP metabolism in these cells, presumably by binding to the same BA receptor, since the effects of the two inducers were not additive.

It has been reported that the metabolism of carbon tetrachloride reduces the cytochrome P-450 content by initiating lipid peroxidation in the adrenal gland (31). However, this is unlikely in the case of BA for the following reasons: antioxidants added to the culture me-

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dium do not prevent the suppression of 17α-hydroxylation by BA (see Fig. 1); 17α-hydroxylase is reportedly insensitive to lipid peroxidation (32); and two enzymes, P-450$_{11\beta}$ and 21-hydroxylase, both of which are highly sensitive to lipid peroxidation, are relatively insensitive to BA. The significance of PAH metabolism in the adrenal is not known; however, the demonstration that PAH suppress specific cytochrome P-450-dependent activities in BAC cells suggests that the toxicity of these compounds is related to interaction with specific targets and not cytotoxicity.

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REFERENCES

1. Tait, J. F., S. A. S. Tait, and J. B. G. Bell. Steroid hormone production by mammalian adrenocortical dispersed cells. *Essays Biochem.* **16**:99–174 (1980).
2. Guenthner, T. M., D. W. Nebert, and R. H. Menard. Microsomal aryl hydrocarbon hydroxylase in rat adrenal: regulation by ACTH but not by polycyclic hydrocarbons. *Mol. Pharmacol.* **15**:719–728 (1979).
3. Colby, H. D., R. C. Rumbaugh, M. L. Marquess, P. B. Johnson, M. R. Pope, and R. E. Stitzel. Strain differences in adrenal xenobiotic metabolism in guinea pigs. *Drug Metab. Dispos.* **7**:270–273 (1979).
4. Colby, H. D., M. L. Marquess, P. B. Johnson, and M. R. Pope. Effects of steroid hormones *in vitro* on adrenal xenobiotic metabolism in the guinea pig. *Biochem. Pharmacol.* **29**:2373–2377 (1980).
5. Montelius, J., D. Papadopoulos, M. Bengtsson, and J. Rydström. Metabolism of polycyclic aromatic hydrocarbons and covalent binding of metabolites to protein in rat adreanl gland. *Cancer Res.* **42**:1479–1486 (1982).
6. Mitani, F. Cytochrome P-450 in adrenocortical mitochondria. *Mol. Cell. Biochem.* **24**:21–43 (1979).
7. Kominami, S., H. Ochi, Y. Kobayashi, and S. Takemori. Studies on the steroid hydroxylation system in adreanl cortex microsomes: purification and characterization of cytochrome P-450 specific for steroid C-21 hydroxylation. *J. Biol. Chem.* **255**:3368–3394 (1980).
8. Nakajin, S., and P. F. Hall. Microsomal cytochrome P-450 from neonatal pig testis: purification and properties of a C_{21} steroid side-chain cleavage system (17α-hydroxylase-$C_{17,20}$ lyase). *J. Biol. Chem.* **256**:3871–3876 (1981).
9. Kominami, S., K. Shinzawa, and S. Takemori. Purification and some properties of cytochrome P-450 specific for steroid 17α-hydroxylation and C_{17}-C_{20} bond cleavage from guinea pig adrenal microsomes. *Biochem. Biophys. Res. Commun.* **109**:916–921 (1982).
10. DuBois, R. N., E. R. Simpson, J. Tuckey, J. D. Lambeth, and M. R. Waterman. Evidence for a higher molecular weight precursor of cholesterol side-chain-cleavage cytochrome P-450 and induction of mitochondrial and cytosolic proteins by corticotropin in adult bovine adrenal cells. *Proc. Natl. Acad. Sci. U. S. A.* **78**:1028–1032 (1981).
11. Privalle, C. T., J. F. Crivello, and C. R. Jefcoate. Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. *Proc. Natl. Acad. Sci. U. S. A.* **80**:702–706 (1983).
12. Faust, J. R., J. L. Goldstein, and M. S. Brown. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured mouse adrenal cells. *J. Biol. Chem.* **252**:4861–4871 (1977).
13. Crivello, J. F., and C. R. Jefcoate. Intracellular movement of cholesterol in rat adrenal cells: kinetics and effects of inhibitors. *J. Biol. Chem.* **255**:8144–8151 (1980).
14. Purvis, J. L., J. A. Canick, J. I. Mason, R. W. Eastabrook, and J. L. McCarthy. Lifetime of adrenal cytochrome P-450 as influenced by ACTH. *Ann. N. Y. Acad. Sci.* **212**:319–343 (1973).
15. Hornsby, P. J. Regulation of cytochrome P-450-supported 11β-hydroxylation of deoxycortisol by steroids, oxygen, and antioxidants in adrenocortical cell cultures. *J. Biol. Chem.* **255**:4020–4027 (1980).
16. Hornsby, P. J. Regulation of 21-hydroxylase activity by steroids in cultured bovine adrenocortical cells: possible significance for adrenocortical androgen synthesis. *Endocrinology* **111**:1092–1101 (1982).
17. Kramer, R. E., J. L. McCarthy, E. R. Simpson, and M. R. Waterman. Effects of ACTH on steroidogenesis in bovine adrenocortical cells in primary culture: increased secretion of 17α-hydroxylated steroids associated with a refractoriness in total steroid output. *J. Steroid Biochem.* **18**:715–723 (1983).
18. Funkenstein, B., J. L. McCarthy, K. M. Dus, E. R. Simpson, and M. R. Waterman. Effect of adrenocorticotropin on steroid 21-hydroxylase synthesis and activity in cultured bovine adrenocortical cells. *J. Biol. Chem.* **258**:9398–9405 (1983).
19. Gospodarowicz, D., C. R. Ill, P. J. Hornsby, and G. N. Gill. Control of bovine adrenal cortical cell proliferation by fibroblast growth factor: lack of effect of epidermal growth factor. *Endocrinology* **100**:1080–1089 (1977).
20. Fahl, W. E., C. R. Jefcoate, and C. B. Kasper. Characteristics of benzo[*a*]pyrene metabolism and cytochrome P-450 heterogeneity in rat liver nuclear envelope and comparison to microsomal membrane. *J. Biol. Chem.* **253**:3106–3113 (1978).
21. Sims, P. Studies on the metabolism of 7-methylbenz[*a*]anthracene and 7,12-dimethylbenz[*a*]anthracene and its hydroxymethyl derivatives in rat liver and adrenal homogenates. *Biochem. Pharmacol.* **19**:2261–2275 (1970).
22. Gustafsson, J. -A., A. Mode, G. Norstedt, T. Hökfelt, C. Sonnenschein, P. Eneroth, and P. Skett. The hypothalamo-pituitary-liver axis: a new hormonal system in control of hepatic steroid and drug metabolism, in *Biochemical Action of Hormones* (G. Litwak, ed.) Vol. 7. Academic Press, New York, 47–89 (1980).
23. Lee, I. P., K. Suzuki, H. Mukhtar, and J. R. Bend. Hormonal regulation of cytochrome P-450-dependent monooxygenase activity and epoxide-metabolizing enzyme activities in testis of hypophysectomized rats. *Cancer Res.* **40**:2486–2492 (1980).
24. Simonian, M. H., M. L. White, and G. N. Gill. Growth and function of cultured bovine adrenocortical cells in serum-free defined medium. *Endocrinology* **111**:919–927 (1982).
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
26. Keller, G. M., C. R. Turner, and C. R. Jefcoate. Kinetic determinants of benzo[*a*]pyrene metabolism to dihydrodiol epoxides by 3-methylcholanthrene-induced rat liver microsomes. *Mol. Pharmacol.* **22**:451–458 (1982).
27. Burke, M. D., H. Vadi, B. Jernström, and S. Orrenius. Metabolism of benzo[*a*]pyrene with isolated hepatocytes and the formation and degradation of DNA-binding derivaties. *J. Biol. Chem.* **252**:6424–6431 (1977).
28. Bumpus, J. A., and K. M. Dus. Bovine adrenocortical microsomal hemeproteins P-450$_{17\alpha}$ and P-450$_{C\text{-}21}$: isolation, partial characterization, and comparison to P-450$_{scc}$. *J. Biol. Chem.* **257**:12696–12704 (1982).
29. Berry, D. L., T. J. Slaga, N. M. Wilson, P. K. Zachariah, M. J. Namkung, W. M. Bracken, and M. R. Juchau. Transplacental induction of mixed-function oxygenases in extra-hepatic tissues by 2,3,7,8,-tetrachlorodibenzo-p-dioxin. *Biochem. Pharmacol.* **26**:1383–1388 (1977).
30. Gehly, E. B., W. E. Fahl, C. R. Jefcoate, and C. Heidelberger. The metabolism of benzo(*a*)pyrene by cytochrome P-450 in transformable and nontransformable C3H mouse fibroblasts. *J. Biol. Chem.* **254**:5041–5048 (1979).
31. Colby, H. D., W. C. Brogan, and P. R. Miles. Carbon tetrachloride-induced changes in adrenal microsomal mixed function oxidases and lipid peroxidation. *Toxicol. Appl. Pharmacol.* **60**:492–499 (1981).
32. Hornsby, P. J., and J. F. Crivello. The role of lipid peroxidation and biological antioxidants in the function of the adrenal cortex. Part 2. *Mol. Cell. Endocrinol.* **30**:123–147 (1983).

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