ACTH- AND CYTOCHALASIN-RELATED CHANGES IN ADRENAL CELL MORPHOLOGY AND CYTOSKELETON

THESIS

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Following 1 hr incubation with ACTH, cytochalasin D or ACTH/cytochalasin, detergent-solubilized mouse adrenal tumor cells cytoskeletal changes were examined using scanning and transmission electron microscopy. Steroid production was also examined.

Control and cytochalasin-treated steroid production was similar; cytochalasin reduced ACTH-stimulated steroid production.

Control cytoskeletons contained microtubule bundles and random arrays of microfilaments, singly and in bundles. Perinuclear microfilaments were found in rounded ACTH-treated cells; microfilament bundles were missing and microtubule bundles radiated from the nuclear area. Treatment with cytochalasin alone, or with ACTH, caused these changes: rounded, stellate morphologies, perinuclear fibrous masses, radiating microtubule bundles, but few microfilaments.

Cytochalasin may inhibit steroid production by altering microfilaments and preventing their retraction during ACTH response.

PART I

ACTH- AND CYTOCHALASIN-RELATED CHANGES IN ADRENAL CELL MORPHOLOGY AND CYTOSKELETON

Background

The Y-1 Cell as a Model System

In 1951 a functional adrenocortical tumor was discovered in a mouse exposed to radiation from the nuclear test, "Operation Green House." The tumor was maintained for six years by transplantation in mice of the LAF-1 strain (Cohen et al., 1957) then put into tissue culture in 1962 (Buonassi et al., 1962). Four years later, a single cultured cell was selected to establish a stable, clonal cell line that retained differentiated steroidogenic capabilities (Yasumura et al., 1966) similar to non-tumor mouse adrenal cells.

The synthesis of steroids in normal rodent adrenal tissue involves enzymes located in two subcellular organelles, the mitochondria and smooth endoplasmic reticulum (Schulster et al., 1976). The initial phase of steroidogenesis requires transport of cholesterol to the inner mitochondrial membrane, where side-chain cleavage occurs (Schulster et al., 1976). Pregnenolone, the product of this reaction, passes from the mitochondria to the smooth membranes of the cytoplasm where two hydroxylation steps occur producing progesterone and deoxycorticosterone (Schulster et al., 1976). The final step in steroid synthesis involves another hydroxylation in the mitochondria and results in corticosterone formation (Schulster et al., 1976). The Y-1 mouse

adrenal cells lack the microsomal 21-hydroxylase activity usually associated with the smooth membranes of the cytoplasm (Kowal, 1970); as a result, a normally minor steroidogenic pathway in rodent adrenals has become important, causing the major secretory products of the Y-1 cell to be 20α-dihydroprogesterone and 11β-20α-dihydroprogesterone (Pierson, 1967).

The Y-1 cells have proved to be a valuable tool for studying the mechanisms regulating steroid production following addition of stimulating agents to cultured cells. Strong evidence has been obtained which indicates that 3',5'-cyclic adenosine monophosphate (cAMP) and cAMP-protein kinase are mediators in the sequence of events leading to steroidogenesis (Sala et al., 1979; Koroscil and Gallant, 1980). Agents known to increase intracellular levels of cAMP and steroid synthesis in the Y-1 cells include ACTH (Kowal, 1970; Donta et al., 1973; Wolff et al., 1973; Koroscil and Gallant, 1980); cholera toxin (Donta et al., 1973; Wolff et al., 1973); bacterial endotoxin (Wolff and Cook, 1975); and adenosine (Wolff and Cook, 1973). Associated with increased steroid production in response to stimulation is a transition from the flat, epithelial morphology of the Y-1 cell to a rounded appearance that extends long, narrow lamellapodial processes to the growth substrate (Donta et al., 1973; Cuprak et al., 1977; Kawaoi et al., 1977). Morphological and biochemical studies suggest an involvement of the complex cellular array of filaments known as the cytoskeleton in steroidogenesis and rounding (McPherson and Ramachandran, 1980; Mattson and Kowal, 1980; Mrotek et al., 1982).

Filaments Comprising the Cytoskeleton

Transmission electron microscopic examination of eukaryotic cells reveals a filamentous cytoskeletal network composed of microfilaments (5-7 nm), intermediate filaments (10 nm), and microtubules (25 nm) (Buckley and Porter, 1967).

Microfilaments

The microfilaments of more than 60 cells have been identified as actin filaments by their specific ability to bind heavy meromyosin (Pollard and Weihing, 1974). In transmission electron micrographs of sectioned tissues, microfilaments are primarily distributed in the region immediately underlying the plasma membrane (Buckley and Porter, 1967), in broad pseudopodial areas (Reaven and Axline, 1973), and within microprojections such as filipodia, lamellapodia and microvilli (Muklerjee and Stachelin, 1971; Tilney and Mooseker, 1971). Cultured cells exhibit two general patterns of microfilament arrangements, namely (a) loosely associated filaments, which are dispersed throughout the cytoplasm, are located in the perinuclear space or are in "ruffled" areas at the edge of motile cells; (b) the highly organized bundles of filaments, called stress fibers, in anchorage-dependent cells (Goldman and Fuller, 1970; Goldman, 1971; Goldman and Knipe, 1972). Functionally, microfilaments (a) form a transitory contractile ring during division of two cells (Fujiwara and Pollard, 1976); (b) accumulate in large quantities beneath the plasma membrane of cells with regions of active phagocytosis (Davies and Stossel, 1977), ruffling or blebbing (Herman et al., 1981); and (c) are observed in

the region between the mitotic spindle poles and chromosomes during mitosis (Sanger, 1975a,b). Microfilaments are also involved in steroid hormone synthesis (Mrotek and Hall, 1975; 1977; Crivello and Jefcoate, 1978) and insulin secretion (Lacy et al., 1968).

Intermediate Filaments

The abundance of 10 nm filaments in a variety of cells is well documented (Bretcher, 1975; Blose and Chacko, 1976; Felix and Stäuli, 1976). Arrays of 8-10 nm filaments have been observed radiating from "synthesis and organizing centers" and terminating in desmosomes in PTK-1 cells (Wang and Goldberg, 1976). In BHK-21 cells, 10 nm filaments are dispersed alone, or in small groups throughout the cytoplasm, often in association with microtubules (Goldman and Knipe, 1972). During cell "spreading" from the rounded to the flattened, fibroblastic shape characteristic of BHK-21 cells, the intermediate filaments are first found to be aggregated in the juxtanuclear region; they then redistribute themselves into the peripheral regions during spreading (Goldman and Fuller, 1970). Similar juxtanuclear aggregates of intermediate filaments form in cells treated with agents causing microtubular breakdown (Robbins and Gonatas, 1967; Goldman, 1971; Holtzer et al., 1975). The most widely discussed roles for intermediate filaments are those of maintaining shape and providing intracellular support in cells (Ferrans and Roberts, 1973; Blose and Chacko, 1976; Cook, 1976). They also appear to function, along with microtubules, in axonal transport (deBrabander et al., 1975; Hoffman and Lasek, 1975), distribution of pigment granules within melanocytes (Moellman and McGuire, 1975),

organelle movement in BHK cells (Goldman, 1971; Goldman and Knipe, 1972), and cell locomotion (Goldman, 1971; Goldman et al., 1973). An involvement of intermediate filaments in steroid hormone secretion has also been suggested (Forbes and Dent, 1974).

Microtubules

Microtubules were discovered in a large number of cell types after the introduction of glutaraldehyde fixation techniques (Sabatini et al., 1963). The typical nine plus two arrangement of cilia, flagella, and some sperm tails depends on the association of microtubules in this pattern (Aiello and Sleigh, 1972; Afzelius, 1959). Centrioles have a somewhat similar characteristic arrangement of microtubules (Aiello and Sleigh, 1952). Centrioles seem to serve as the nucleating site for production of the microtubules which constitute the mitotic spindle (McIntosh et al., 1975). In animal cells, microtubules are frequently grouped in long, parallel lines within extensions of the plasma membrane such as lamellapodia, filipodia, microvilli or pseudopodia (Tilney and Gibbons, 1969). Similar microtubular arrangements are found in dendritic processes of melanophores (Bikle et al., 1966; Moellman and McGuire, 1975). Microtubules are generally thought to function in chromosomal movement in cell division (Pickett-Heaps, 1969), maintenance of cell form (Tilney, 1971), cell motility (Robbins and Gonatas, 1967), sensory transduction (deBrabander et al., 1975; Hoffman and Lasek, 1975), and intracellular transport (Goldman and Fuller, 1970; Goldman, 1971).

Cytoskeletal Involvement in Adrenocortical Steroid Production

The evidence for the involvement of cytoskeletal elements in steroid production by adrenal cells is indirect and, in some studies, conflicting. In general, these studies were conducted using various cultured adrenal cell lines which were incubated either with drugs which prevent formation of microtubules and microfilaments, or with antibodies reacting against cytoskeletal proteins. The evidence for the participation of these elements in steroid production and for the presence of cytoskeletal elements in steroid-producing adrenal cells will be examined in the following paragraphs.

Stimulation of steroidogenesis in adrenal cells by ACTH can be blocked by inhibitors of microtubule or microfilament formation (Mrotek and Hall, 1975, 1977; Cortese and Wolff, 1978; Crivello and Jefcoate, 1978; Ray and Strott, 1978; McPherson and Ramachandran, 1980). Anti-actin antibodies also inhibit ACTH-stimulated steroidogenesis when introduced into the adrenal cell (Hall et al., 1979). It has been suggested that these inhibitors and antibodies affect the cytoskeletal components involved in the transport of cholesterol from the cytoplasm to the mitochondrion where steroidogenesis is initiated (Mrotek and Hall, 1975, 1977; Crivello and Jefcoate, 1978; Hall et al., 1979; Mrotek et al., 1982).

Mrotek and Hall (1978) showed that various inhibitors of microtubule formation had no effect on the acute response of the adrenal cell to ACTH and that microfilament-disorganizing agents inhibited steroid production (Mrotek and Hall, 1975, 1977). A number of studies

indicate that, under certain conditions, inhibitors of microtubule and microfilament formation stimulate adrenal steroidogenesis (Temple and Wolff, 1973; Ray and Strott, 1978; Cortese and Wolff, 1978; Clark and Shay, 1979; 1981; McPherson and Ramachandran, 1980). Although incubation of normal or Y-1 adrenal tumor cells with the microtubule-inhibiting agent, colchicine, stimulate steroid production (Temple and Wolff, 1973), the elevation in steroid production occurs at a slower rate than that associated with ACTH. Incubation of mouse adrenal tumor cells with the microfilament inhibitors, cytochalasin B or D, in the presence of serum-containing medium resulted in a rapid increase in steroid production (Cortese and Wolff, 1978; McPherson and Ramachandran, 1980). However, the mechanism of action of cytochalasin stimulation is different from that of ACTH. For instance, ACTH stimulation was independent of the presence of serum, while cytochalasin stimulation was dependent on serum, and ACTH increased intracellular cAMP while cytochalasin did not (Cortese and Wolff, 1978). In addition, the stimulatory effect of the cytochalasins was not observed in non-tumor rat adrenal cells (McPherson and Ramachandran, 1980). The indirect evidence that cytoskeletal elements are involved in basal- and ACTH-stimulated steroid production appears to depend on the drug used, the adrenal cell line and the incubation conditions, with or without ACTH.

Few workers have examined directly adrenal cells for cytoskeletal elements or changes in the cytoskeleton following treatment with anti-cytoskeletal agents and ACTH. The actin-containing microfilaments of the cytoskeleton were first identified in sections of rat and hamster

adrenals using fluorescent anti-actin auto-antibodies from human hepatitis patients (Gabbiani et al., 1975). Primary and clonal cultures of Y-1 mouse adrenal tumor cells also contain thin cytoskeletal filaments (Mattson and Kowal, 1980; Mrotek et al., 1982). The thin filament protein, actin (Pollard and Weihing, 1974), has been isolated from the Y-1 adrenal cells (McPherson and Ramachandran, 1980; Hall et al., 1981). The observation of microtubules in cultured Y-1 adrenal cells was first reported by Mrotek and Hall (1978), and later confirmed by others (Clark and Shay, 1979; 1981; Mattson and Kowal, 1980; Mrotek et al., 1982). Fluorescent antibodies against the microtubule protein, tubulin, also demonstrate the existence of microtubules in mouse adrenal tumor cells (Clark and Shay, 1979, 1981). The only morphological evidence for the existence of intermediate cytoskeletal filaments in adrenal cells came from the identification of 100 Å filaments in Triton X-100 Y-1 cell cytoskeletal residues (Mrotek et al., 1982). Three studies reported rearrangements in the thin filaments and microtubules of Y-1 adrenal cells incubated with ACTH (Clark and Shay, 1979, 1981; Mattson and Kowal, 1980; Mrotek et al., 1982); incubation of Y-1 cells with cytochalasin in the presence or absence of ACTH also resulted in thin filament rearrangements (Mrotek et al., 1982).

Summary and Proposed Research

Based on the preceeding review the following statement will summarize our current knowledge regarding the morphological and physiological response of the adrenal cell to ACTH. The adrenal cell rounds in response to incubation with ACTH; in non-adrenal cells, rounding

involves rearrangement of cytoskeletal elements. Cytoskeletal elements are composed of microfilaments, intermediate filaments and microtubules. Steroid production is stimulated by ACTH, by certain drugs which prevent microtubule formation and by drugs inhibiting the formation of microfilaments. Under certain conditions, the anti-microfilament drugs cytochalasin B and D will inhibit ACTH-stimulated steroid production. However, these drug-related studies provided indirect evidence that various components of the adrenal cell cytoskeleton may be involved in rounding and in steroid production. Studies directly associating the ACTH- and cytochalasin D-related changes in steroidogenesis, whole cell morphology and individual components of the cytoskeleton, are also needed. In the paper which follows, clonal cultures of Y-1 mouse adrenal tumor cells were chosen as a model system to examine total steroid production, cell morphology and cytoskeletal relationships before and after treatment with ACTH, cytochalasin D or ACTH/cytochalasin D.

PART II

ACTH- AND CYTOCHALASIN D-RELATED CHANGES IN Y-1 ADRENOCORTICAL CELL CYTOSKELETON AND CELL SURFACE TOPOGRAPHY

Introduction

A variety of endocrine cells which normally exhibit a flattened shape when maintained in monolayer culture respond to their trophic hormone with a change in morphology termed rounding. For example, granulosa cells maintained in monolayer culture round in response to follicle-stimulating hormone (Lawrence et al., 1979). In addition, adrenocorticotropin (ACTH) induces rounding in cultured rat (O'Hare and Neville, 1973), human (Simonian and Gill, 1981) and mouse Y-1 (Yasumura et al., 1966; Cuprak et al., 1977) adrenocortical cells. Rounding is characterized by a retraction of the thin peripheral cytoplasmic areas toward the nucleus, an adherence of the plasma membrane to underlying cytoplasmic structures, an elevation of the nuclear region from the growth substrate, and an extension of long, narrow lamellapodial processes from the nuclear region (Miller et al., 1976; Kawaoi et al., 1977; Lawrence et al., 1979; Mattson and Kowal, 1980).

The Y-1 mouse adrenal tumor cell line is accepted as a model system for studying steroid hormone production following ACTH stimulation (Kowal, 1970, Temple and Wolff, 1973; Mrotek and Hall, 1975,

1977; Mattson and Kowal, 1980). This line has been used extensively to study the effects of ACTH, 3',5'-cyclic adenosine monophosphate (cAMP), cholera toxin, or other agents causing increased levels of intracellular cAMP, increased steroid production and cellular rounding (Masui and Garren, 1971; Donta et al., 1973; Cuprak et al., 1977; Kawaoi et al., 1977). The morphological changes induced by ACTH and other stimulating agents appear to reflect a rearrangement of the cytoskeleton (microtubules and microfilaments) within the adrenocortical cell (Clark and Shay, 1979, 1981; Mattson and Kowal, 1980; Mrotek et al., 1982) and occur concomitantly with an increase in steroid output (Cuprak et al., 1977; Kawaoi et al., 1977; Mattson and Kowal, 1980). However, there have been few attempts to examine directly the cytoskeletal elements of adrenal cells, and the relationship(s) between the morphological and steroidogenic responses to ACTH are not yet clearly defined. Kawaoi and co-workers (1977) reported that cells which round when stimulated with ACTH show a greater steroidogenic response than cells which do not exhibit a change in shape. Further, cytochalasins B and D, mold metabolites which inhibit microfilament function (Rathke et al., 1975; Tannenbaum, 1978), cause morphological changes in Y-1 adrenal tumor cells similar to those induced by ACTH (Mrotek et al., 1982), and they both stimulate and inhibit steroidogenesis in Y-1 cells (Mrotek and Hall, 1975, 1977; Cortese and Wolff, 1978; Crivello and Jefcoate, 1978; Clark and Shay, 1979; McPherson and Ramachandran, 1980; Hall et al., 1981).

The present studies were conducted to enable further examination of the steroidogenic and morphological changes induced by ACTH and

cytochalasin D in Y-1 adrenocortical tumor cells. High Pressure (Performance) Liquid Chromatography (HPLC) was used to measure steroids released into the incubation medium. General changes in cell morphology were observed with scanning electron microscopy (SEM). In order to directly correlate morphological changes observed by SEM with alterations in the cytoskeleton, adrenocortical tumor cells were treated with ACTH in the presence or absence of cytochalasin D and extracted with Triton X-100. Triton X-100 dissolved in a buffer that stabilizes the cytoskeleton (Schliwa and van Blerkom, 1981) extracts the majority of the cytoplasmic ground substance, but leaves the insoluble cytoskeletal structures, nuclear area and polyribosomes relatively intact (Lenk et al., 1977; Bell et al., 1978; Schliwa and van Blerkom, 1981). The extracted cells were then examined by transmission electron microscopy (TEM). The results reported here indicate that cytochalasin D inhibits steroidogenesis by specifically disrupting microfilaments and preventing them from retracting in response to ACTH stimulation of steroid production.

Materials and Methods

Cell Culture

Mouse adrenal tumor cells (Y-1) obtained from the American Type Culture Collection (Rockville, MD) were maintained in Eagle's Minimum Essential Medium (GIBCO, Santa Clara, CA) containing 12.5% horse serum (GIBCO), 2.5% fetal calf serum (Kansas City Biologicals, Lenexa, KS), 0.01% L-glutamine (Sigma Chemical Co., St. Louis, MO) and 50 µg/ml gentamycin sulfate (Sigma). Stock cultures of cells were grown on 25

cm^2 tissue culture plates (Corning, Fort Worth, TX) in a humidified, 5% carbon dioxide/95% air atmosphere at 37° C. The cells used in these experiments were from the 53rd through the 56th population doublings.

Incubation of Cells with Experimental Treatments

Twenty-four hours before the experiment, cells were subcultured into six-well plates (Costar, Cambridge, MA) containing 100 mesh gold grids (E. Fullam, Schenectady, NY) which were previously coated with Formvar (E. Fullam) and carbon, then sterilized with ultraviolet (UV) radiation. Cells were maintained in serum-containing medium until the beginning of the experiment. The medium was then removed, and the cells were washed twice with serum-free medium (SFM) and pre-incubated for two, half-hour periods in 2 ml of SFM. This medium was discarded and the cells were again washed with SFM. Cells were then incubated for one half hour in 2 ml SFM and the medium was collected, along with an SFM wash, to measure basal steroid production. Cells were then incubated for 1 hr in 2 ml of SFM to which ACTH, cytochalasin D or ACTH plus cytochalasin D were added. At the completion of the experiment, the medium was withdrawn, the cells were washed with 2 ml SFM and the pooled wash and incubation medium was used to assay steroid production. Incubations were carried out with duplicate plates of cells, and the experiment was carried out twice.

Experimental Solutions

A stock solution of ACTH (Sigma) dissolved in 0.1 M acetic acid was prepared on the day of the experiment. This stock solution was added to SFM to prepare an incubation medium with a 10^{-6} M final ACTH

concentration. Cytochalasin D (Sigma) was dissolved in dimethylethylsulfoxide (DMSO) and then diluted with SFM to a final concentration of 10^{-6} M. The final concentrations of DMSO or acetic acid did not exceed 0.01%. Appropriate volumes of vehicles were added to the medium of cells that were not treated with ACTH or cytochalasin D so that the concentrations of DMSO and acetic acid were equal in all incubations.

Steroid Measurement

The steroid content of the incubation medium was assayed by HPLC. HPLC was performed using a Waters Associates (Milford, MA) High Performance Liquid Chromatograph consisting of a Model 6000A solvent pump and a Model 450 variable wavelength detector. Steroids were extracted from the incubation medium with 5 ml methylene dichloride containing 250 ng of dexamethasone (Sigma) as an internal standard, and the extract was evaporated to dryness under nitrogen. Steroids were then dissolved in methanol and separated by HPLC on a C_{18}-μBondapack reverse phase column in an isocratic system of 65% methanol (u/u) flowing at a rate of 1.5 ml per min under a pressure of 2500 psi. Absorbance at 240 nm was used to detect Δ^4-3-ketosteroids; they were quantitated by comparison of absorbance peak areas with those of authentic standards. Standards included 11β-,20α-dihydroxypregn-4-en-3-one (11-DHP, Medical Research Council Steroid Reference Collection), 11β-hydroxyprogesterone (11-P, Sigma), 20α-hydroxypregn-4-en-3-one (20-DHP, Sigma), progesterone (P, Sigma). Two separate experiments provided the samples for assay.

Extraction of Cells and Preparation for Electron Microscopy

Cells cultured on gold grids were washed with 2 ml of buffer [65 mM 1,4-piperazinediethane sulfonic acid (PIPES), 25 mM N-2-hydroxyethylpeperinen-N-2-ethane sulfonic acid (HEPES), 10 mM ethylene glycolbis (β-aminoethyl ether) N,N,N',N'-tetracetate (EGTA) and 2 mM magnesium sulfate, pH 6.9] and then extracted by a modification of the procedure of Schliwa (1980). Briefly, the cells were incubated for 3 min in the above wash buffer containing 1% Triton X-100 (Sigma). The grids were then washed twice with buffer, and incubated for 20 min in 1% glutaraldehyde in wash buffer. Fixative was removed, grids were rinsed twice and a 3 min staining with 0.5% osmium tetroxide was initiated. The cellular residues were dehydrated with increasing concentrations of acetone, and critical point dried. All solutions used in preparing the cytoskeletal residues were at room temperature. The cytoskeletal residues obtained from each treatment were carbon coated, then examined using a JOEL 1000C High Voltage Transmission Electron Microscope (Boulder, CO).

Preparation of Cells for Scanning Electron Microscopy

The cells grown on gold grids were fixed for 30 min in 2.9% glutaraldehyde in Hank's buffer (5.4 mM potassium chloride, 4.4 mM potassium phosphate, 6.5 mM sodium phosphate, 0.14 M sodium chloride, 0.5 mM glucose, pH 7.4). The grids were washed three times with Hank's buffer, then postfixed 3 min in 1% osmium tetroxide in water. Grids were then washed twice with water and cell residues were dehydrated in a graded series of acetone solutions, transferred to a critical point

dryer (The Bowmar Co., Tacoma, WA) and dried through carbon dioxide. The preparations were coated with gold-palladium and examined by SEM using an ETEC Autoscan SEM (courtesy, Department of Anatomy, Texas College of Osteopahtic Medicine, Fort Worth, TX).

Results

Steroid Production

Steroid production by Y-1 adrenal cells treated with cytochalasin D or ACTH alone, and in combination with cytochalasin D, is summarized in Table 1. Cells incubated in serum-free media containing 0.5 U/ml ACTH caused total steroids to be produced at a rate six-fold greater

Table 1

Steroid Compounds Secreted by Y-1 Adrenal Cells in Response to Treatments Containing ACTH and Cytochalasin D[a]

Treatment	*Steroids*					
	11-DHP[b]	11-P	P	20-DHP	Unknown Compound	Total HPLC Steroids
Control	ND	ND	16.1	19.8	21.0	56.9
ACTH	147.4	33.9	ND	18.6	162.4	362.3
CD	ND	ND	12.2	13.7	14.0	39.9
ACTH + CD	ND	ND	ND	8.6	22.3	30.9

[a]ng/ml incubation medium (total incubation volume 3 ml). Values represent the mean of two experiments.

[b]Abbreviations are: 11-DHP, 11β-,20α-dihydroxypregn-4-en-3-one; 11-P, 11β-hydroxyprogesterone; P, progesterone; 20-DHP, 20α-dihydroprogesterone (20α-hydroxypregn-4-en-3-one); CD, cytochalasin D; ND, not detected.

than did control cells. Cytochalasin D (10^{-6} M) almost completely inhibited ACTH-stimulated steroid production, but appeared to have little effect on basal steroid output. An unidentified compound, having a retention time of 30 min, was detected in the medium from each treatment (Table 2). In response to ACTH the production of this compound increased; an increase did not occur when cells were treated with a combination of ACTH and cytochalasin D.

Table 2

HPLC Retention Times for Steroid Compounds Secreted by Y-1 Adrenal Cells[a]

Compound	*Retention Time (min)*
Dexamethasone (internal standard)	5.0
11β-20α-dihydroxypregn-4-en-3-one	7.0
11β-hydroxyprogesterone	8.5
Progesterone	17.0
20α-dihydroprogesterone	18.0
Unidentified Compound	30.0

[a]Compounds were detected at 240 nm; flow rate was 1.5 ml of 65% methanol (v/v) per min.

Morphological Examination of Y-1 Cells

Scanning microscopy of whole cells.--A variety of polymorphic shapes could be observed when control cultures of Y-1 adrenal tumor cells were examined with scanning electron microscopy [Figure 1a]. Since the peripheral margins of the majority of the cells were closely

adherent to the growth substrate, the cells had a flattened, spread-out appearance. Except for an occasional bleb or microvillus, the surface of the cells had few topographic features.

Y-1 cells stimulated with ACTH for one hour developed a stellate morphology [Figure 1b]. The peripheral margins of a majority of the cells retracted toward the nucleus, leaving long, narrow lamellapodial processes radiating from the rounded area to the growth substrate. The cell surface developed an increased number of microvilli and blebs.

Treatment of Y-1 cells with cytochalasin alone or in combination with ACTH induced retraction of the peripheral cell margins resulting in formation of an elevated central nuclear area [Figures 1c, d]. Lamellapodia connecting this nuclear area to the growth substrate gave the cell a stellate shape. The central elevated nuclear area developed blebs and an increased number of microvilli. The stellate cells, produced with either of the cytochalasin treatments, co-existed with a population of cells which assumed an appearance described for non-adrenal cytochalasin-treated cells as discoid, apolar "pancake"-shaped [Figure 1d].

Scanning microscopy of extracted cells.--Detergent-extracted Y-1 control cells examined with SEM had a flattened, polymorphic shape similar to that of whole unextracted cells [Figure 2a]. A Triton-resistant fibrous net, which surrounded the now visible ovoid nucleus, comprised the matrix and gave the extracted cell its shape.

The stellate morphology of ACTH-treated cells was maintained after extraction with Triton X-100 [Figure 2b]. The nucleus was embedded in a mound of fibrous material with the fibrous remains of the lamellapodia

extending from the top of the nuclear area.

An elevated nucleus resting on Triton-resistant cytoskeletal elements was observed in both the cytochalasin-treated controls [Figure 2c] and ACTH/cytochalasin-treated cells [Figure 2d]. The lamellapodia in both treatments seemed to be broader in width and may contain coarser fibers.

Transmission microscopy of extracted cells.--The detergent-insoluble cytoskeletal residue of control cells consisted of fibrous elements corresponding in size to thin filaments (5-7 nm) and microtubules (25 nm) distributed within a fibrous net [Figure 3a]. In addition, numerous polyribosomes were associated with these fibrous elements. Many thin filaments distributed throughout the fibrous net were accumulated into bundles to form stress fibers. Microtubules could be observed throughout the extracted cell, occasionally associating in groups of tubules which oriented parallel to one another.

Y-1 cells stimulated with ACTH showed a general retraction of the peripheral margins of the cell [Figure 3b]. The perinuclear fibrous material contained thin filaments and it appeared more electron dense due to its withdrawal toward the nuclear area. Most of the fibrous components in the lamellapodia correspond to microtubules. Polyribosomes were especially prominent in the nuclear mass, although a few groups were also found in the lamellapodia.

The fibrous mass surrounding the central nuclear area of extracted cytochalasin-, or ACTH and cytochalasin-, treated cells was too thick to allow penetration by the electron beam; the size characteristics of the cytoskeleton in this area could not be determined [Figure 3c, d].

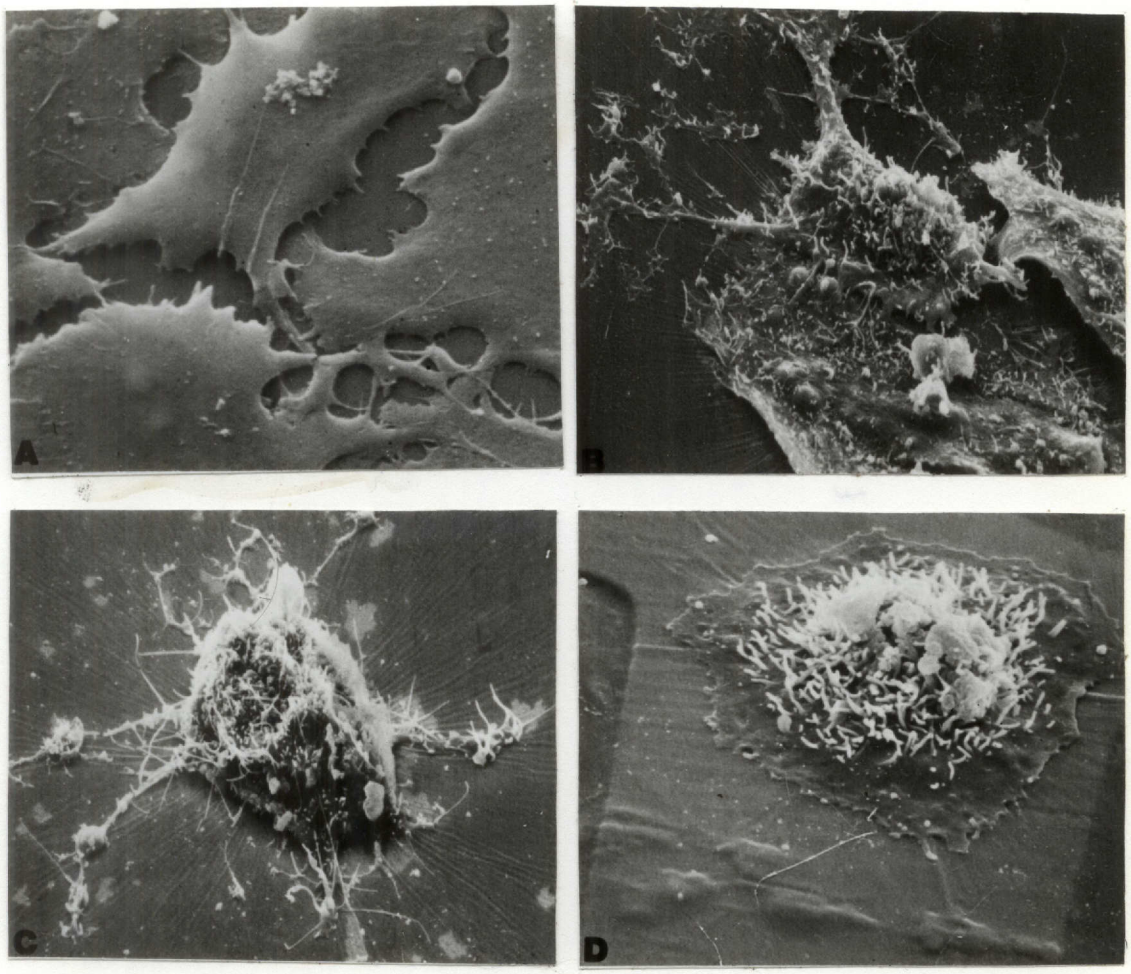

Figure 1. Scanning electron micrographs of intact Y-1 adrenocortical cells. X2000

a) non-treated; b) ACTH-treated; c) cytochalasin-treated; d) ACTH with cytochalasin treatment

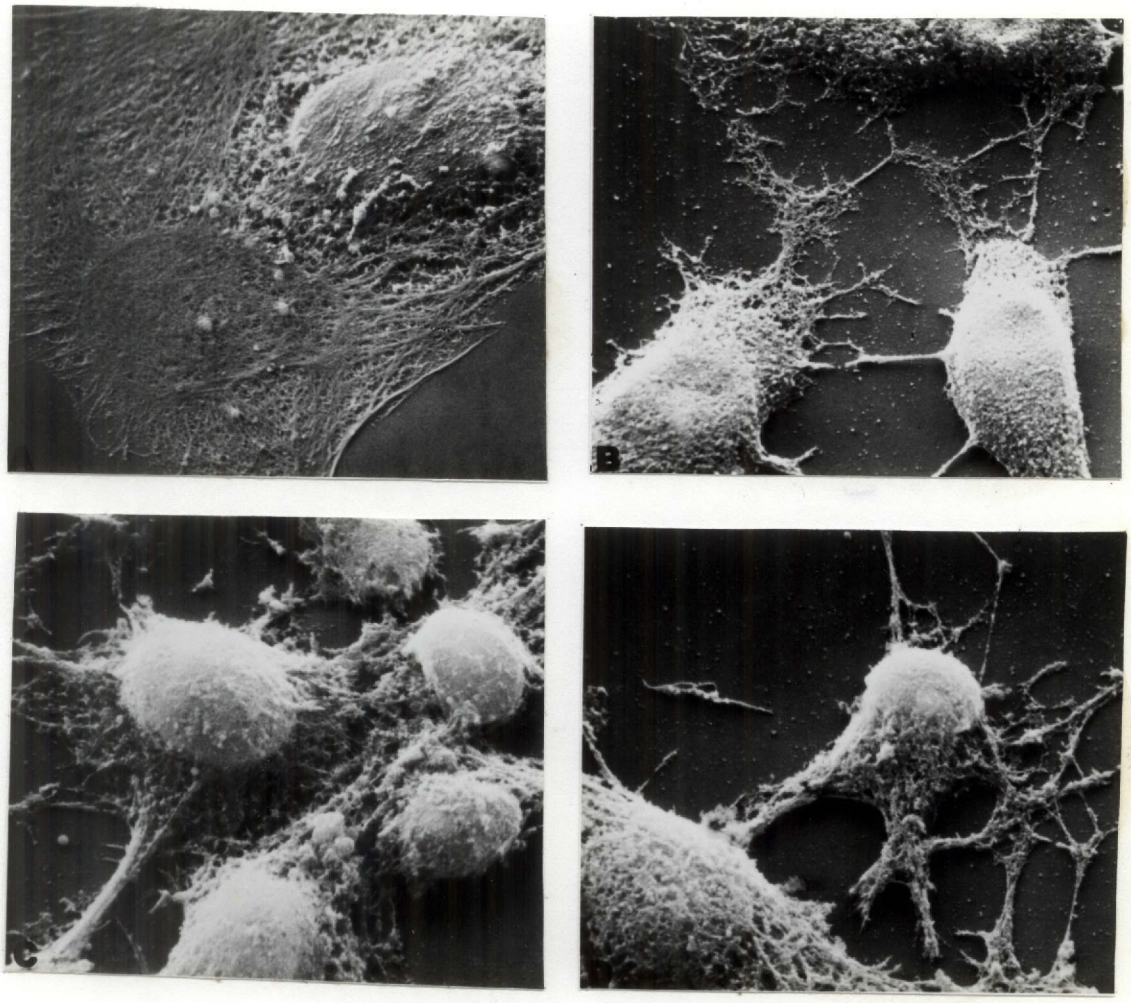

Figure 2. Scanning electron micrograph of Triton X-100 extracted Y-1 adrenocortical cells. X2000 a) non-treated; b) ACTH-treated; c) cytochalasin-treated; d) ACTH with cytochalasin treatment

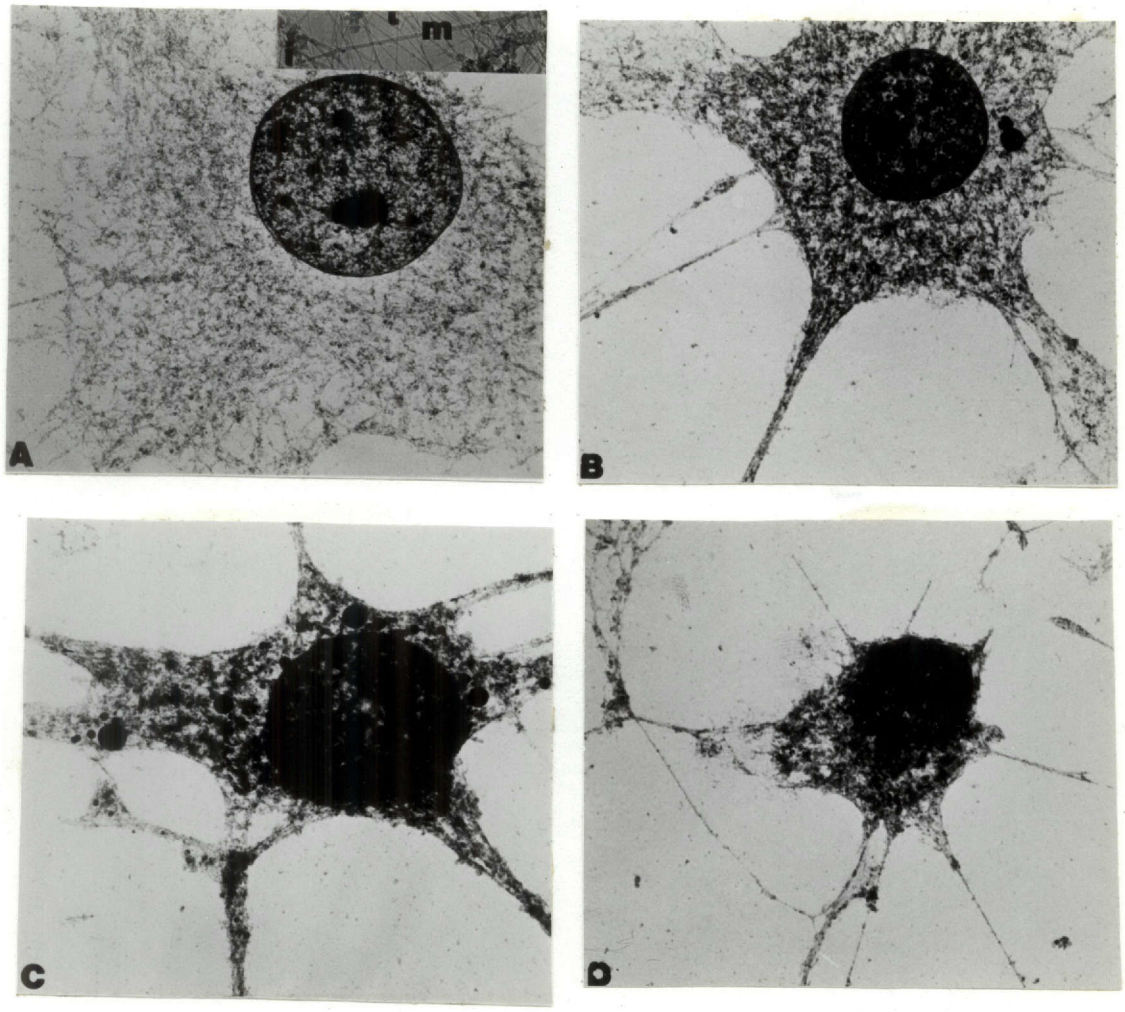

Figure 3. Transmission electron micrograph of Triton X-100 extracted Y-1 adrenocortical cells. X2000

a) non-treated [Insert: high magnification of microtubules (m) and thin filaments (t). X40,000]; b) ACTH-treated; c) cytochalasin-treated; d) ACTH with cytochalasin treatment

Examination of the lamellapodia radiating from the central area revealed few thin filaments and numerous microtubules. Polyribosomes appeared to be uniformly distributed along the length of the lamellapodia.

Discussion

It is generally accepted that cytoskeletal components are involved in mediating the morphological changes which occur in cultured adrenal cells in response to ACTH (Mattson and Kowal, 1980; Cheitlin and Ramachandran, 1981; Mrotek et al., 1982). In addition, most (Crivello and Jefcoate, 1978; Hall et al., 1979; Mrotek et al., 1982), but not all (Cortese and Wolff, 1978), studies of cultured adrenal cells suggest that the cytoskeleton plays a role in ACTH-stimulated steroidogenesis. Yet, few investigators have attempted to directly associate cytoskeletal ultrastructure with the steroidogenic response of adrenal cells to ACTH. In the present study, the relationship between cytoskeletal changes and steroid production were examined in Y-1 adrenal cells treated with ACTH and cytochalasin D, either alone or in combination. Cells were examined using both scanning and transmission electron microscopy; total steroids secreted into the incubation medium were measured by HPLC.

Y-1 adrenal cells changed topography and substrate attachment in response to ACTH and cytochalasin treatments. Scanning electron microscopy showed that Y-1 cells incubated with ACTH or cytochalasin develop a central "rounded" area with lamellapodia attached to the growth substrate. During ACTH stimulation, Y-1 cells were examined

using time-lapse photomicrography; while certain peripheral regions of the cell retracted during rounding, the lamellapodia appeared to grow outward from other locations on the periphery (Voorhees and Mrotek, 1981, unpublished observations). These morphological changes occurred in conjunction with increased blebbing and microvilli formation on the cell surface. The increased blebbing was more pronounced in response to cytochalasin than to ACTH. Similar morphological and topographical changes have been observed in a number of other cell types following cytochalasin treatment (Godman et al., 1975; Davies and Stossel, 1977). Several investigators have also observed similar topographical changes in Y-1 cells following ACTH stimulation of steroid production (Cuprak et al., 1977; Kawaoi et al., 1977; Mattson and Kowal, 1980).

The morphological changes in Y-1 adrenal cells were compared with intracellular cytoskeletal changes by using transmission electron microscopy to examine cells extracted with Triton X-100. Microfilament bundles, normally observed in control cells, were not apparent in ACTH-stimulated cells; rather, the lamellapodia contained parallel arrays of microtubules. In addition, individual microfilaments were apparent and these appeared to be retracted toward the nucleus. These observations agree with those of previous studies in which the effects of ACTH were examined in whole cell mounts and thin-sectioned Y-1 cells (Mattson and Kowal, 1980; Mrotek et al., 1982). Cells which are flat-growing in tissue culture depend on organized bundles of microfilaments, called stress fibers, to maintain close attachment to the growth substrate (McNutt et al., 1973; Pollack et al., 1975). On the other hand, rounded cells appear to contain fewer stress fibers, and these fibers

reform as the rounded cell begins to flatten (Bragina et al., 1976). The present finding that lamellapodia contain parallel arrays of microtubules is consistent with the observations that microtubules form in response to treatment of Y-1 adrenal cells with ACTH or cAMP (Clark and Shay, 1981), and that there is an extension of lamellapodia from the cell periphery of the Y-1 cell in response to ACTH (Voorhees and Mrotek, 1981, unpublished observations). The morphological and cytoskeletal changes observed following ACTH treatment of Y-1 cells suggest that the rounding of these cells during stimulation coincides with the loss of microfilament bundles. However, it is not clear whether the individual microfilaments near the nuclear area of ACTH-treated cells represent relocated single filaments or microfilaments derived from the filament bundles.

Microfilaments and stress fibers were also absent in the lamellapodia and thin peripheral regions of extracted cells treated with cytochalasin D, or ACTH and cytochalasin D. The present observation is consistent with other observations that cytochalasins disrupt microfilament attachments to plasma membranes (Weber et al., 1976) and block actin monomer polymerization into microfilaments or to existing microfilament fragments (Hartwig and Stossel, 1976; MacLean et al., 1978; Lin et al., 1980; MacLean-Fletcher and Pollard, 1980). In the cytochalasin-treated cells of the present study, a thick, electron-dense amorphous mass in the perinuclear region prevented the identification of specific cytoskeleton filaments in this site. Davies and co-workers (1973) suggested that retraction of the cell periphery after cytochalasin treatment results from displacement of cortical actin filaments;

such a displacement would allow the outer cytoplasmic regions of the cell to flow inward to form a dense perinuclear mass and leave filopodia as the only peripheral cell surface-substrate attachment. When fluorescent antibodies reacting with actin were used to study non-adrenal cells treated with cytochalasin, actin was found in perinuclear masses (Weber et al., 1976; Woda et al., 1977). However, in preliminary studies at this laboratory, fluorescent anti-actin antibodies revealed numerous star-shaped points randomly distributed throughout the cytoplasmic area of the cytochalasin-treated Y-1 cells, suggesting that the cytochalasin had either precipitated or depolymerized the actin-containing filaments, thereby preventing perinuclear accumulations. This pattern of actin precipitation in response to cytochalasin is similar to the arborized matts of microfilaments observed beneath the cell membrane of non-adrenal cells treated with cytochalasins (Weber et al., 1976; Temmink and Spiele, 1981). Although the failure to identify microfilaments in the cytochalasin-treated cytoskeletal residues of this study could also result from Triton X-100 releasing microfilaments, or stress fibers, having no substrate or cytoskeletal attachments, the preliminary evidence obtained using fluorescent anti-actin antibodies argues that much of the actin was precipitated by the cytochalasin treatment.

It is still unclear whether the ultrastructural changes induced by ACTH are directly related to steroidogenesis. Both ACTH and cytochalasin produced similar topographical and morphological changes, but only ACTH stimulated steroidogenesis. In addition, ACTH caused cytoskeletal fibers to retract toward the nucleus, while cytochalasin

seemed to cause microfilaments to precipitate. The inhibitory effect of cytochalasin on ACTH-stimulated steroidogenesis may reflect an ability to either disrupt microfilaments or to prevent their formation rather than an ability to nonspecifically affect Y-1 cell activities. Data from others using Y-1 cells incubated in cytochalasin-containing media lacking serum, indicates that basal steroid production, synthesis of ATP and protein, cholesterol uptake into the cell, and conversion of exogenous pregnenolone to end-product steroids are not affected by the inhibitor. In addition, the conversion of cholesterol to pregnenolone by isolated adrenal mitochondria or by preparations of purified adrenal mitochondrial side-chain cleavage enzyme also is not affected (Mrotek and Hall, 1977). These observations further corroborate the view that the effects of cytochalasins are specific.

Cytochalasins appear to inhibit ACTH-stimulated transport of cholesterol into the mitochondria of Y-1 adrenal cells (Mrotek and Hall, 1977; Hall et al., 1981) and rat adrenal cells (Crivello and Jefcoate, 1978). Hall and co-workers (1981) also noted that cytochalasin D inhibited the effects of LH and cAMP on mitochondrial cholesterol uptake in mouse Leydig cells. These observations, together with those of the present study suggesting that cytochalasin disrupts cellular microfilaments and prevents them from rearranging in response to ACTH, are consistent with the hypothesis that microfilaments in some way moderate steroidogenesis, perhaps regulating the transport of cholesterol to the mitochondria.

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