CYTOCHALASIN-STIMULATED STEROIDOGENESIS
FROM HIGH DENSITY LIPOPROTEINS

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ABSTRACT

The cytochalasins stimulate steroid secretion of Y-1 adrenal tumor cells two- to threefold. The order of potencies is cytochalasin E > D > B, but the maximum response is the same and always less than with ACTH. Like that with ACTH, the stimulation has a rapid onset, is easily reversible, is inhibited by cycloheximide and aminoglutethimide, and occurs at a stage before pregnenolone. Although the cytochalasins, like ACTH, produce cell rounding, it is shown that this morphological change is not necessarily coupled to steroidogenesis. Unlike ACTH, cytochalasin B does not measurably increase cellular levels of cAMP at concentrations that lead to maximal steroidogenesis.

The cytochalasin B-induced stimulation of steroidogenesis, unlike the short-term ACTH effect, fails to occur in the absence of serum. This lack of response can be corrected by even low concentrations of human high density lipoproteins (HDL) but not by low density lipoproteins (LDL). We, therefore, propose that cytochalasin B enhances the availability of cholesterol bound to HDL for steroidogenesis by Y-1 adrenal cells.

KEY WORDS Y-1 adrenal tumor cell rounding · ACTH · cAMP · colchicine · vinblastine · aminoglutethimide

The participation of microtubules and microfilaments in the secretory process has been widely proposed. To a large extent, this proposal is based on the effects of drugs that alter these components of the cytoskeleton: colchicine, podophyllotoxin and vinblastine in the case of microtubules, and the cytochalasins in the case of microfilaments (1, 41, 45). Although in most cases the above drugs inhibit secretion (45), in some cases they enhance it (3, 5, 7, 15, 18, 19, 36). For instance, in ACTH-responsive adrenal tumor cells in culture (Y-1 cells), antimicrotubule agents increase steroid production (39). It seemed of interest, therefore, to ascertain whether or not the cytochalasins, which also can cause intracellular organelle rearrangement, might similarly enhance steroidogenesis in this tumor cell line. In this paper, we report that cytochalasins are indeed able to increase steroid secretion in Y-1 cells. In contrast to ACTH-induced stimulation, the action of these macrolides is totally dependent upon the presence of some serum factor(s), probably the high density lipoproteins (HDL).1 They seem to provide a new mechanism by which adrenal steroid production can be influenced.

1 Abbreviations used in this paper: 1-24ACTH, the N-terminal 24 residues of ACTH (synacthen); DMSO, dimethylsulfoxide; LDL, low density lipoproteins; and HDL, high density lipoproteins.
MATERIALS AND METHODS

Chemicals

The cytochalasins, obtained from Aldrich Chemical Co. (Milwaukee, Wis.), were dissolved in dimethylsulfoxide (DMSO); appropriate dilutions were made with 10% DMSO/water (vol/vol) before the compounds were added to the incubation medium in which the final concentration of DMSO was always lower than 0.3%. ^24ACTH, i.e., the peptide containing the first 24 N-terminal residues of the native hormone, was a generous gift of Ciba Corp. (Summit, N. J.). Colchicine, cycloheximide, proglucagon, and 20α-dihydroprogesterone were obtained from Sigma Chemical Co. (St. Louis, Mo.). 11β-hydroxy-20α-dihydroprogesterone was synthesized in the Chemistry Department (Westfield College, Hampstead, London) and was kindly supplied by Dr. J. L. Goldstein (University of Texas Southwestern Medical School, Dallas, Tex.). Vinblastine sulfate was obtained from Eli Lilly & Co. (Indianapolis, Ind.), aminoethylthiimide from Ciba, and human low density lipoproteins (LDL) and human high density lipoproteins (HDL) were gifts from Drs. B. Brewer and Robert W. Mahley of the National Heart, Lung and Blood Institute (Bethesda, Md.).

Cells

The ACTH-responsive Y-1 mouse adrenal tumor cell line, introduced in culture by Buonassisi et al. (4), was obtained from the American Type Culture Collection (Rockville, Md.). Cells were maintained in Ham’s F-10 medium supplemented with 12.5% horse serum and 2.5% fetal calf serum, penicillin (124 μg/ml) and streptomycin (270 μg/ml). Cells were grown in 75 cm² Falcon flasks in a water-saturated, 5% CO₂, 95% air atmosphere at 37°C with medium changes every 2–3 days. Cultures were divided with 0.25% Viskase solution (Grand Island Biological Co., Grand Island, N.Y.).

Measurement of Steroid Secretion

All the experiments were performed when the monolayers were nearly confluent. The dishes used in a single experiment were always from a single subculture. The assays were performed in the same serum-containing medium in which the cells were grown and were run for 1 h at 37°C unless otherwise stated. Steroid secretion into the culture medium was measured fluorometrically as previously described (39).

The Y-1 cells have been shown to secrete chiefly 20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone (23). Steroids were extracted with methylene chloride, dried and dissolved in ethanol for analysis by thin-layer chromatography in four solvents (39), and were detected by quenching of fluorescence. In all the solvents, only 11β-hydroxy-20α-dihydroprogesterone was found, whether the cells were incubated in control conditions, with cytochalasin B, or with ACTH. Results are expressed as equivalents of 20α-dihydroprogesterone, since this compound is commercially available and could be used as a standard in fluorescence measurements. The fluorescence yield of 11β-hydroxy-20α-dihydroprogesterone is approx. 1.6 times that of 20α-dihydroprogesterone and, therefore, we overestimated by this factor the amounts of steroid produced.

For cAMP determinations, confluent Y-1 cells were washed twice with Ham’s F-10 medium at 37°C and incubated with ^24ACTH or cytochalasin B. Reactions were stopped with cold 5% trichloracetic acid, the cells were harvested by scraping with a rubber policeman, and the precipitated cells and protein were centrifuged. The supernatant solution was extracted four times with 4 vol of water-saturated ethyl ether, and the aqueous phase was used for cAMP assays according to Gilman (14). Protein was determined with crystalline bovine albumin as standard (29).

RESULTS

Stimulation of Steroidogenesis in Y-1 Cells by ^24ACTH and the Cytochalasins

The Y-1 cells were very sensitive to ^24ACTH, half-maximal stimulation occurring at approx. 4 × 10⁻¹¹ M ^24ACTH (Fig. 1). All the cytochalasins tested were able to increase steroid secretion (Fig. 1). The half-maximal stimulatory concentrations were 0.3 μM, 0.8 μM, and 4 μM for cytochalasins E, D, and B, respectively. The maximum stimulation attained by all analogues was the same but always less than with ^24ACTH. In general, the cytochalasins increased steroidogenesis by 2.5–3.5-fold and ^24ACTH by 5–16-fold. The degree of stimulation by ^24ACTH varied largely because of differences in basal steroidogenesis. DMSO had no effect on steroidogenesis up to 0.5% (Table I). On a concentration basis, the order of potencies was cytochalasin E > cytochalasin D > cytochalasin B (Fig. 1). This observation is consistent with the known potency of cytochalasins in other systems (6, 30). The effect on steroidogenesis is not related to inhibition of glucose transport, since cytochalasin B is a strong inhibitor of glucose uptake, whereas cytochalasins D and E are much less potent and, in some systems, totally ineffective in this respect (21, 30, 37). In addition, the lack of effect of 2-deoxyglucose (Table I) shows that under our conditions, i.e., in short incubation experiments, inhibition of glucose transport does not influence the rate of steroid production. In the experiments reported below, we used cytochalasin B but the same effects could be detected as well with its analogues.
Effect of ACTH and Cytochalasin B on the Shape of Y-1 Cells

A striking feature of Y-1 cells is their morphologic response to steroidogenic stimuli. When exposed to ACTH (46), cholera toxin (9, 44), endotoxins (42), cAMP (26, 39), or adenosine (25, 43), the flat epithelial form of the unstimulated cell (Fig. 2A) changes to a rounded, refractile one that extends long processes and is separated from its neighbors in confluent cultures (Fig. 2C and D). All cytochalasins caused complete rounding of Y-1 cells that was indistinguishable from rounding produced by \( 1^{24}\text{ACTH} \) (Fig. 2C and D). These morphological changes were complete in 40–60 min and were reversed upon removal of the stimulators in 10–20 min. Full rounding occurred at maximally stimulatory concentrations of \( 1^{24}\text{ACTH} \) (1 x 10 \(^{-6}\) M and above) and, for the cytochalasins, over the same concentration range at which they increase steroidogenesis. ACTH concentrations that increase steroidogenesis to the same extent as the cytochalasins did not, however, cause significant rounding (Fig. 2B).

Time-Course of Steroidogenesis

Unlike the steroidogenic response of Y-1 cells to antimicrotubule agents (39), the stimulation by cytochalasin B appeared to be instantaneous and exhibited no significant lag (Fig. 3, left panel). This time-course is like that produced by \( 1^{24}\text{ACTH} \). The rate of steroid output remained virtually linear for 1 h but fell off thereafter. Moreover, the stimulation produced by cytochalasin B was readily reversible when the drug was removed from the culture dish since the rate of steroidogenesis returned to control values after ten min (Fig. 3, right panel). The washing maneuver itself had no effect if cytochalasin B was again added. The rapid onset and the easy reversibility is typical of other effects of the cytochalasins as well as of their binding to cell or plasma membranes (34, 38).

Site of Cytochalasin B Action on Steroid Metabolism

It is generally agreed that ACTH acts on the cleavage of the cholesterol side chain, increasing the rate of pregnenolone production, and does not
FIGURE 2  Effect of ^24ACTH and cytochalasin B on the shape of Y-1 cells. Y-1 cells were incubated for 1 h with the compounds and then photographed by phase-contrast microscopy. Under each picture is indicated the steroid production by the dishes. (A) Control; (B) ACTH, $1 \times 10^{-10}$ M; (C) cytochalasin B, 8 $\mu$M; and (D) ACTH, $1 \times 10^{-9}$ M. (A–D): $\times$100.

have major early effects on the subsequent conversion of pregnenolone to steroid end products (22, 23). This is true also for cytochalasin B. When pregnenolone was added to the incubation medium, the synthesis of 20α-dihydroprogesterone differed little in control, ACTH- or cytochalasin B-treated cells (Table II). In order to confirm that cytochalasin B acts on the conversion of cholesterol to pregnenolone, we studied the effect of aminoglutethimide, a drug known to block this conversion without affecting many other parameters of Y-1 cell metabolism (24). As shown in Table III, aminoglutethimide inhibited steroidogenesis in controls, ACTH- and cytochalasin B-treated cells.

A labile protein is believed to be required to mediate the action of ACTH, since cycloheximide, at concentrations at which it blocks protein synthesis, inhibits the ACTH effect on steroidogenesis (8, 12). Cytochalasin B stimulation of steroidogenesis was similarly sensitive to cycloheximide inhibition (Fig. 4, left panel). Upon removal of cycloheximide, the inhibition of steroidogenesis was rapidly reversed and the initial rate was reestablished (Fig. 4, right panel). The experiment with the above compounds thus shows that cytochalasin B- and ACTH-stimulated steroidogenesis share a number of general characteristics.

Other agents known to increase steroid production by Y-1 cells are the antimicrotubule drugs, colchicine, podophyllotoxin, and vinblastine (39). That the antimicrotubular drugs and the cytochalasins act on different sites was suggested by the fact that the former exhibit a time lag of 6–8 h before exerting any effect, whereas the latter act instantaneously. Additional evidence for this is provided by the fact that the effects of cytochalasin B and colchicine, or cytochalasin B and vinblas-
**Figure 3** Time-course of steroid secretion and reversibility of the cytochalasin B effect. *Left panel:* Four dishes for each group were incubated with 10 ml of standard medium, and 1.0 milliliter was withdrawn at each time-point. The cumulative steroid secretion is indicated in the graph. Cytochalasin B was 6 μM and ACTH 3 × 10⁻¹¹ M. *Right panel:* Three control dishes (O-O) and six dishes containing 8 μM cytochalasin B (●-●) were incubated for 1 h and then washed. The control dishes were reincubated with medium only, whereas the cytochalasin B group was split into two sets, each containing three dishes, incubated with medium only (O-O), or with 8 μM cytochalasin B (●-●).

**Table II**

| Condition                | Pregnenolone (10 μg/ml) | 20α-Dihydroprogesterone μg/mg protein/h* |
|--------------------------|-------------------------|-------------------------------------------|
| Control                  | −                       | 0.061 ± 0.006                             |
| Cytochalasin B (10 μM)   | −                       | 0.13 ± 0.016                              |
| ACTH (5 × 10⁻⁹ M)        | −                       | 0.71 ± 0.068                              |
| Control                  | +                       | 6.6 ± 1.2                                 |
| Cytochalasin B (10 μM)   | +                       | 6.9 ± 0.95                                |
| ACTH (5 × 10⁻⁹ M)        | +                       | 7.1 ± 0.55                                |

* Y-1 cells were incubated for 1 h in 5 ml of medium without (−) or with (+) pregnenolone (10 μg/ml). Every group contained three dishes. Pregnenolone fluorescence was less than 2% of 20α-dihydroprogesterone fluorescence; and since ~25% of the added pregnenolone was converted to steroid end products in the incubation time, its value was not subtracted from the fluorescence measurements.

* Mean ± SD.

**Table III**

| Condition                | Aminoglutethimide (0.2 mM) | 20α-Dihydroprogesterone μg/mg protein/h* |
|--------------------------|-----------------------------|-------------------------------------------|
| Control                  | −                           | 0.069 ± 0.001                             |
| Control                  | +                           | 0.01 ± 0.008                              |
| ACTH (3 × 10⁻⁹ M)        | −                           | 0.71 ± 0.023                              |
| ACTH (3 × 10⁻⁹ M) +      | +                           | 0.021 ± 0.017                             |
| Cytochalasin B (6 μM)    | −                           | 0.22 ± 0.033                              |
| Cytochalasin B (6 μM) +  | +                           | 0.01 ± 0.006                              |

* Y-1 cells were incubated without (−) or with (+) aminoglutethimide and the stimulators at the stated concentration. Three dishes per group each measured in duplicate.

* Mean ± SD.

... was approximately additive (Table IV). Under our experimental conditions, ACTH alone was more powerful than the combination of antimicrotubule agents and cytochalasin B.
Effect of cycloheximide on cytochalasin B-induced steroid secretion. Left panel: Dishes were preincubated with cycloheximide at the concentrations indicated for 30 min, washed, and reincubated with the same concentration of inhibitor with (○-●) or without 8 μM cytochalasin B (□-□). Right panel: Three dishes for each group were incubated with the compounds stated below, washed, and reincubated at 40 min (arrow); in the second incubation, cycloheximide was omitted, the other conditions being the same as in the first incubation: Curve 1: 24 ACTH; curve 2: first incubation 24 ACTH and cycloheximide, second incubation 24 ACTH; curve 3: cytochalasin B; curve 4: first incubation cytochalasin B and cycloheximide, second incubation cytochalasin B; and curve 5: control. The concentrations of the compounds were: ACTH, 3 × 10^{-9} M; cytochalasin B, 8 μM; and cycloheximide, 10 μM.

Effect of cAMP Concentration

It is generally held that the steroidogenic response to ACTH is mediated by an early burst of cAMP production. We, therefore, investigated cAMP levels after exposure of Y-1 cells to cytochalasin B. As shown in Fig. 5, at no time during the first twenty min after exposure to 8 μM cytochalasin B was there a significant change in the cellular level of cAMP. This is in marked contrast to the cAMP levels found after exposure of Y-1 cells to 24ACTH. Concentrations of 1 × 10^{-10} M 24ACTH produced a rapid doubling of the cellular cAMP content, and large 24ACTH concentrations led to a 6–7-fold increase by the first time interval tested (2 min). However, since 1 × 10^{-10} M 24ACTH produced a much larger increase in steroidogenesis than did cytochalasin B, e.g., 12-fold vs. 3-fold in Fig. 1, a proportional increase in cAMP levels might be too small to detect. Nevertheless, these data clearly show that substantial increases in cellular cAMP concentrations do not occur when steroidogenesis is enhanced by cytochalasin B.

Mediation of the Cytochalasin B Effect on Steroidogenesis by HDL

Mrotek and Hall have reported (31, 32) that, in contrast to the findings presented here, cytochalasin B inhibited ACTH-stimulated steriodo-

### Table IV

| Condition                  | 20α-Dihydroprogesterone (μg/mg protein/h) |
|----------------------------|------------------------------------------|
| Control                    | 0.099 ± 0.003                            |
| Colchicine (5 μM)          | 0.41 ± 0.031                             |
| Vinblastine (5 μM)         | 0.39 ± 0.020                             |
| Cytochalasin B (8 μM)      | 0.25 ± 0.005                             |
| Colchicine + Cytochalasin B| 0.57 ± 0.005                             |
| Vinblastine + Cytochalasin B| 0.51 ± 0.021                           |
| ACTH (3 nM)                | 1.26 ± 0.10                              |

Three dishes were incubated for each group. The dishes containing vinblastine or colchicine were preincubated with the drug for 8 h, then washed twice with the medium as with the other dishes, and reincubated with the same drug as in the preincubation.

* Mean ± SD.

genesis in Y-1 cells. Although they used higher concentrations of the macrolide than we did, a major feature of their study was that steroidogenesis was assayed in the absence of serum. We, therefore, studied the role of serum on the effect of cytochalasin B. In the experiments reported below, Y-1 cells, grown in the presence of 12.5% horse serum and 2.5% fetal calf serum, were washed and preincubated for 2 or 3 h with serum-
free Ham's F-10 medium. They were then washed again and incubated for assay purposes. As shown in Table V, the absence of serum in no way impeded the stimulation of steroidogenesis by $^{24}\text{ACTH}$. On the other hand, cytochalasin B was totally ineffective in the absence of serum. Nevertheless, cytochalasin B was still able to induce rounding to the same extent as that seen in the presence of serum. This suggested that serum contained a factor necessary for stimulation of steroidogenesis by cytochalasin B. In view of the critical role of lipoproteins in adrenal cholesterol metabolism (2, 11, 17), we investigated the role of the two major cholesterol-carrying lipoproteins on the response of Y-1 cells to cytochalasin B. As shown in Table V, like serum, HDL readily restored the ability of cytochalasin B to stimulate steroidogenesis in serum-free Y-1 cells. By contrast, even large concentrations of LDL failed to restore this effect of cytochalasin B.

The amounts of HDL used in Table V are consistent with the concentrations that might be expected from the amounts of serum used under our standard conditions. If HDL concentrations in serum range from 1 to 2 mg/ml (13), then media containing 15% serum would be expected to contain 150-300 $\mu$g/ml of HDL. The effect of HDL concentration is depicted in Fig. 6. Lower concentrations of the lipoprotein are required to elicit the cytochalasin B response than are needed to enhance basal steroidogenesis. However, even when large concentrations of HDL are provided, stimulation of steroidogenesis by cytochalasin B can still be demonstrated.

DISCUSSION

The Y-1 cells have proved to be a helpful tool for the study of regulation of steroid secretion because of their high sensitivity to agents known to increase the intracellular levels of cAMP, such as ACTH (46), cholera toxin (9, 44), bacterial endotoxins (42), and adenosine (25, 43). Several years ago, it was shown that antimicrotubule agents, although not affecting cAMP levels, increased steroid production, and it was suggested that microtubules might act as a barrier that restricts the access of cholesterol to the mitochondria (39). In this paper, we report that the cytochalasins also increase the rate of steroid production by Y-1 cells apparently without the intervention of cAMP. The cytochalasins disrupt subplasmalemmal microfilaments (30, 40), interact with the cell membrane (27, 34, 37, 38), inhibit glucose and small molecule diffusion through the cell membrane (10, 16, 20, 21, 28), and have several morphologic as well as biochemical effects in different cell lines (30, 40).

Two of the known mechanisms of cytochalasin action were tested in the present system:

(a) Glucose transport—That enhancement of steroidogenesis produced by cytochalasin B is not due to changes in glucose transport was shown by the findings that (a) cytochalasin D and cytochalasin E, which are much less potent inhibitors of glucose uptake than cytochalasin B, were, on a concentration basis, more potent than cytochalasin B; and (b) the inhibition of glucose transport by 2-deoxyglucose did not affect steroidogenesis. We have no information on the transport of other small molecules.
TABLE V
Mediation of the Cytochalasin B Effect on Steroidogenesis by High Density Lipoproteins

| Experiment | Condition | Incubation medium | 20α-Dihydroprogesterone (μg protein/h) |
|------------|-----------|-------------------|---------------------------------------|
| I          | Basal     | Ham's F-10 (no serum) | 0.068 ± 0.021                        |
|            | Cytochalasin B (8 μM) | "                   | 0.073 ± 0.023                        |
|            | ACTH (3 nM) | "                   | 0.58 ± 0.08                          |
| II         | Control   | Ham's F-10 (no serum) | 0.11 ± 0.013                         |
|            | Cytochalasin B (8 μM) | "                   | 0.12 ± 0.036                         |
|            | Control   | Ham's F-10 + 15% horse serum | 0.15 ± 0.045                      |
|            | Cytochalasin B (8 μM) | "                   | 0.37 ± 0.045                         |
|            | Control   | Ham's F-10 + LDL 600 μg/ml | 0.14 ± 0.003                      |
|            | Cytochalasin B (8 μM) | "                   | 0.17 ± 0.010                         |
|            | Control   | Ham's F-10 + HDL 200 μg/ml | 0.16 ± 0.041                      |
|            | Cytochalasin B (8 μM) | "                   | 0.33 ± 0.037                         |

Y-1 cells, grown in Ham's F-10 containing 12.5% horse serum and 2.5% fetal calf serum, were washed and preincubated for 2 h with serum-free Ham's F-10, washed again, and then incubated either with Ham's F-10 alone or with Ham's F-10 containing horse serum, human HDL, or LDL at the stated final concentrations. LDL and HDL concentrations are as micrograms protein per milliliter.

* Mean ± SD for three dishes per group each assayed in duplicate.

Figure 6 Mediation of cytochalasin B effect on steroidogenesis by human HDL. Y-1 cells, grown in Ham's F-10 containing 12.5% horse serum and 2.5% fetal calf serum, were washed twice and preincubated for 3 h in serum-free Ham's F-10, washed again and incubated for 1 h with Ham's F-10 containing the indicated concentrations of human HDL. Three dishes were incubated for each concentration of HDL with 0.1% DMSO (O-O) or with 8 μM cytochalasin B (●-●).

(b) Morphology—Although rounding of Y-1 cells is a characteristic response to steroidogenic stimuli, we have been able to separate these phenomena completely by: (a) low 1-24ACTH concentrations (Fig. 2 B); (b) rounding without steroidogenesis in the case of local anesthetics (33, 35, and unpublished observations); and (c) rounding without steroidogenesis produced by cytochalasin B in the absence of serum. Thus, cell rounding does not necessarily lead to steroidogenesis.

The cytochalasins are only partial agonists of steroidogenesis when compared to ACTH. Nevertheless, their effect resembles that of ACTH in several ways. Both cytochalasin B and ACTH act before the pregnenolone step in the biosynthetic pathway; their effect is instantaneous and is inhibited by cycloheximide and aminogluthethimide. We were, therefore, surprised to discover that cytochalasin B was totally ineffective in the absence of serum, a condition that does not affect ACTH stimulation over the short time interval employed in these studies. Hence, serum factor(s) appear to mediate the cytochalasin effect on steroidogenesis. The cholesterol used for steroid synthesis in our short incubation experiments may derive from three sources: (a) Newly synthesized cholesterol—this seems to be a minor fraction of cellular cholesterol and provides little substrate for steroid synthesis unless the cholesterol stores are severely depleted (17, 23). (b) Cholesterol stores—these seem to be sufficient to sustain 1-24ACTH stimulation under our conditions since ACTH is effective in the absence of serum. Cholesterol stores utilized for 1-24ACTH stimulation were, apparently, not accessible to cytochalasin B. (c) Extracellular, lipoprotein-bound cholesterol—this can be a substrate for steroidogenesis (2, 11, 17), and facilitation of the transfer of cholesterol...
from lipoprotein into the cell could, conceivably, increase steroidogenesis. Whether the best source for cholesterol is LDL or HDL is a matter of some controversy (2, 11, 17). Under our conditions, HDL, and not LDL, was required for the demonstration of the cytochalasin B effect. We, therefore, propose that cytochalasin B acts by enhancing the availability of cholesterol bound to HDL for steroidogenesis.

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