J. steroid Biochem. Vol. 36, No. 5, pp. 493–499, 1990
Printed in Great Britain

0022-4731/90 $3.00 + 0.00
Pergamon Press plc

FURTHER CHARACTERIZATION OF THE INHIBITORY EFFECT OF MONENSIN ON ADRENAL STEROIDOGENESIS*

BEHLING CHENG,[1,2,3]† IRIS A. HORST[1] and JEROME KOWAL[1,3]

[1]Department of Medicine and [2]Department of Physiology and Biophysics, Case Western Reserve University School of Medicine and [3]Veterans Administration Medical Center, Cleveland, OH 44106, U.S.A.

(*Received* 8 *December* 1989)

Summary—We have previously reported that treatment of cultured mouse adrenal tumor cells with 0.6–1.2 μM monensin, a monovalent carboxylic ionophore, results in disruption of the organized structure of the Golgi complex. This is associated with an inhibition of adrenocorticotropic hormone (ACTH) or dibutyryl cAMP-stimulated steroidogenesis and impairment of mitochondrial cholesterol side-chain cleavage activity. The present report describes further investigations regarding possible mechanisms for the inhibition. Monensin inhibits both synthesis of fluorogenic steroids and incorporation of [^{14}C]acetate into the end-product steroid 11β,20α-dihydroxy-4-pregnen-3-one. Supplementation of monensin-treated cells with 25-hydroxycholesterol, a readily available substrate for steroidogenesis, does not reverse the inhibitory effect on the reaction. The incorporation of L-[^{35}S]methionine into trichloroacetic acid precipitable proteins in the isolated mitochondria of monensin-treated cells is inhibited approximately by 40%, whereas the inhibitory effect on the proteins in the cell homogenate is marginal. These findings suggest that a deficiency of newly synthesized proteins in mitochondria, rather than the availability of the substrate cholesterol, may be the primary factor causing impairment of steroidogenesis.

INTRODUCTION

In 1938, Reese and Moon[1] observed that, following hypophysectomy of the rat, the Golgi complex of the adrenal cortex became atrophic, whereas stimulation of hypophysectomized rats with ACTH-induced hypertrophy of the adrenal Golgi complex. In contrast, the Golgi complex of the adrenal medulla failed to show any consistent change. Subsequently, ACTH-induced hypertrophy of the Golgi complex has been observed not only in rat adrenal cortex [2], but also in cultures of rat fetal adrenocortical cells [3] and mouse adrenal tumor (Y-1) cells [4]. Administration of aminoglutethimide to rats, an inhibitor of the mitochondrial cholesterol side-chain cleavage reaction, induces an accumulation of digitonin-precipitable cholesterol in the Golgi complex of rat adrenal fasciculata cells [5]. Treatment of rats with α-amanitin, an inhibitor of RNA transcription, induces clusters of microvesicles with dense content in the Golgi *trans*-area of adrenal fasciculata cell [6]. We have shown that, using acid phosphatase as a marker, the region of Golgi-endoplasmic reticulum-lysosome (GERL) of Y-1 cells is dramatically activated by ACTH [7]. The Golgi complex in the adrenal cortex has been implicated in the secretion of steroids, transport of steroid intermediates, production of lysosomes, and lipid droplet formation (for a review see [8]).

Monensin is a well characterized monovalent carboxylic ionophore which disrupts the Golgi complex through perturbation of monovalent cation gradients in the organelle of mammalian cells (for a review see [9]). We have used monensin to investigate the possible involvement of the Golgi complex in the regulation of steroidogenesis of Y-1 cells. The key findings of our initial studies [10] are summarized as follows. Treatment of cells with monensin (0.6–1.2 μM) results in disruption of the organized architecture of the Golgi complex of both

*Presented in part as an abstract at the *3rd Annual Symposium of the Ohio Physiology Society* (1988).

†To whom correspondence should be addressed, at the VA Medical Center-Laboratory K102.

Abbreviations: ACTH, adrenocortocotropic hormone; db-cAMP, dibutyryl-cAMP; HEPES, hydroxyethyl piperazine ethanesulfonic acid; HPLC, high-performance liquid chromatography; monensin, 2-[5-ethyltetrahydro-5-[tetrahydro-3-methyl-5-[tetrahydro-6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-2H-pyran-2-yl]-2-furyl]-2-furyl]-9-hydroxy-β-methoxy-α,γ,2,8-tetramethyl-1,6-dioxaspiro[4.5]decane-7-butyric acid; *P*450, total cytochrome *P*450 regardless of specificity; *P*45011β, cytochrome *P*450 which is specifically involved in 11β steroid hydroxylation; $P450_{scc}$, cytochrome *P*450 which is specifically involved in cholesterol side-chain cleavage reaction; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

unstimulated and ACTH-stimulated cells into irregular vacuoles and diminishes vesicular activity associated with the organelle. Concomitant with the disruption is the inhibition of both basal and ACTH or db-cAMP-stimulated steroidogenesis. There is no apparent effect on mitochondrial morphology. Mitochondrial cholesterol side-chain cleavage activity is impaired in the organelles isolated from cells previously stimulated with ACTH in the presence of monensin, but monensin has no apparent direct effect on the activity in isolated mitochondria. We have also demonstrated [11] that the disruption of the Golgi complex is reversible; reorganization of the Golgi structure is associated with full restoration of steroidogenic activity of cells.

Independently, Matsuoka *et al.*[12] also reported that ACTH- and angiotensin II-stimulated aldosterone synthesis by rat adrenal capsular cells *in vitro* is inhibited by monensin. The present report presents additional studies directed at further characterizing the inhibitory effect of monensin on steroidogenesis in cultured Y-1 cells.

EXPERIMENTAL

Materials

$ACTH_{1-24}$ (Cortrosyn) was obtained from Organon (West Orange, N.J.). Authentic 11β,20α-dihydroxy-4-pregnen-3-one was kindly donated by Professor D. N. Kirk, Steroid Collections (London, England). Monensin and other authentic lipids described in the text were purchased from Sigma (St Louis, Mo.). Si-C18 octadecyl precoated TLC plate and HPLC-grade acetonitrile and methanol, and dichloromethane were purchased from J. T. Baker (Phillipsburg, N.J.). Radiochemicals: [2-^{14}C]acetic acid and L-[^{35}S]methionine were purchased from New England (Boston, Mass) and Amersham (Arlington Hts., Ill.), respectively. Except for glucose and $NaHCO_3$ (J. T. Baker), the medium components for cell growth were purchased from Gibco (Grand Island, N.Y.).

Cell culture

The methods of Y-1 cell culture and tumor transplantation have been detailed previously [7, 10, 11, 13–16]. In brief, cells were plated in 60 × 15 mm plastic culture plates and grown in minimum essential medium (MEM) at 37°C in an atmosphere of 5% carbon dioxide. The MEM was prepared by mixing 3 l of distilled water (purified by Millipore Water Systems), 30.0 g of minimum essential medium powder, 6.6 g of $NaHCO_3$, 35 ml of a penicillin (10,000 U/ml)-streptomycim (10,000 μg/ml) solution, 30 ml of a solution containing 10 mM non-essential amino acids, 30 ml of a 200 mM α-glutamine solution, and 45 ml of a glucose solution (100 mg/ml). The medium was further supplemented with 13.4% (v/v) gamma globulin-free horse serum and 2.3% (v/v) fetal calf serum.

Experimental protocols

Before each experiment, the medium of confluent cultures was replaced with serum-free MEM and the cells were incubated in this medium overnight to minimize the possible effect of sera. All experiments were carried out in PBS-RH medium, pH 7.5, which was prepared by mixing an equal portion of Gey's phosphate-buffered saline and Ringer's solution buffered with HEPES [14]. For stimulation, a concentration of ACTH of 100 mU/ml was used to produce a maximal effect on steroidogenesis unless specified otherwise. To study steroidogenesis utilizing endogenously synthesized cholesterol, cells were labeled with [^{14}C]acetate, 1.3×10^6 cpm/plate, for 18 h prior to stimulation with ACTH. To study the levels of *de novo* protein synthesis in cells or mitochondria, cells were labeled with L-[^{35}S]methionine (40 μCi/ml). The other experimental conditions are detailed in the legends of figures and tables.

Determination of fluorogenic steroids

The methodology for assaying fluorogenic steroids has been previously documented by this laboratory [15]. In brief, the culture medium was extracted with 5-fold dichloromethane. Aliquots of dichloromethane containing steroids were mixed with 2 ml of a sulfuric acid-ethanol solution, 70:30 (v/v). After incubation at room temperature for 60 min, fluorescence of the mixture was measured at an excitation wavelength 475 nm and emission wavelength 525 nm in an Aminco-Bowman Spectrophotometer. Authentic 20α-hydroxy-4-pregnen-3-one was used as standard.

Determination of [^{14}C]acetate incorporated into 11β,20α-dihydroxy-4-pregnen-3-one by TLC

The steroid in each extract was purified by a reversed phase TLC system using Si-C18 octadecyl precoated TLC plates (20 × 20 cm) and a solvent-mixture containing acetonitrile:water, 75:25 (v/v). The R_f of 11β,20α-dihydroxy-4-pregnen-3-one was 0.60, whereas the R_fs of other steroid intermediates (progesterone, pregnenolone, 20α-hydroxy-4-pregnen-3-one, and 11β-hydroxyprogesterone) were 0.29–0.50. The R_fs of hydroxycholesterol derivatives such as 25-hydroxycholesterol, 22(*R*)-hydroxycholesterol, and 22(*S*)-hydroxycholesterol were 0.05–0.07. Cholesterol, cholesteryl oleate, squalene, lanosterol, and phospholipids (L-α-phosphatidylinositol, L-α-phosphatidylcholine, L-α-phosphatidylserine, L-α-phosphatidylethanolamine, cardiolipin, and sphingomyelin) remained at the origin in this solvent system. Therefore, this reversed-phase TLC method is suitable for separation of steroid products from labeled cholesterol and other lipids. Authentic 11β,20α-dihydroxy-4-pregnen-3-one and the culture samples were run in parallel on the same TLC plate. The location of the authentic compound was detected by charring after a brief spray with a sulfuric acid-dichromate reagent. The corresponding untreated

areas in the channels of the culture samples were removed and counted in a Searle Tracer Analytic counter.

Determination of 11β,20α-dihydroxy-4-pregnen-3-one by HPLC

A specific reversed-phase HPLC system for this purpose has recently been reported from this laboratory [16]. Briefly, 2.5 μg 17α-hydroxyprogesterone, the internal standard, in 10 μl ethanol was added to 1 ml culture medium immediately prior to extraction. The culture medium was extracted at room temperature with a 5-fold volume of dichloromethane by vigorously vortexing for 1 min. After centrifugation, the aqueous phase was removed and the organic phase was washed with 0.5 ml distilled water. After further centrifugation, 4 ml of the organic phase was removed and dried under a stream of nitrogen. The dried residues were dissolved in 100 μl methanol and 50 μl was injected into a HPLC system as described below.

The HPLC system (Waters Assoc., Milford, Mass) used consisted of dual pumps (model 510), a computer (model 680), a u.v. detector (model 450), and a C-18 μ Bondapak reverse-phase column (3.9 mm i.d. × 30 cm) connected with a guard column. An integrator/recorder (Shimadzu, model CR3A, Tokuj, Japan) was used for data analysis. The mobile phase employed was a solvent-mixture composed of acetonitrile:methanol:water, 35:11:54 (v/v/v). Isocratic flow-rate was set at 1 ml/min. The separation of steroids was monitored at 254 nm (u.v.). The steroid content was calculated from a standard curve which demonstrated a linear relationship between the area-ratio of 11β, 20 α-dihydroxy-4-pregnen-3-one peak to the internal standard peak and the known amount of authentic steroid added prior to extraction.

Determination of L-[^{35}S]methionine incorporated into cellular and mitochondrial proteins

Our established protocol was described previously [17]. Cells were scraped out from culture plates with a rubber policeman and were homogenized in a buffered solution containing 10 mM Tris, pH 7.4, and 8.6% sucrose (w/v) at 4°C. Mitochondria were then prepared from cell homogenate as Ref. [10]. Aliquots of cell homogenate and mitochondrial suspension were taken to determine protein content by a modification of Lowry's procedure [18]. The radioactivity of TCA-precipitated cellular, and mitochondrial proteins on No. 540 Whatman paper filter was counted in 5 ml Liquifluor.

Statistical analysis

The statistical significance of the difference between the sample means was determined by unpaired Student's *t*-test.

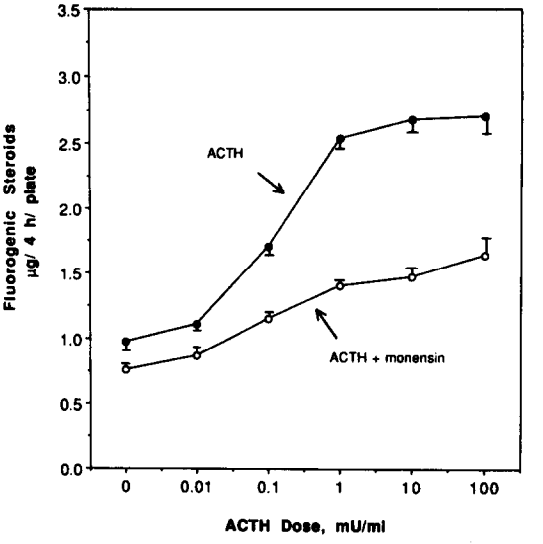

Fig. 1. The effect of monensin on ACTH dose-dependent steroidogenesis. Cells were stimulated with varied ACTH doses (0.01–100 mU/ml) in the presence or absence of monensin (1.2 μM) for 4 h. Data are presented as means ± SD of 4 determinations.

RESULTS

As shown in Fig. 1, monensin (1.2 μM) inhibited steroidogenesis of Y-1 cells to a greater extent in the presence of 1-100 mU/ml ACTH than in the presence of lower doses of the hormone. Prolonged incubation of cells with monensin up to 24 h (Fig. 2), or exposure of cells to higher concentrations of monensin up to 60 μM (Fig. 3), further increased the inhibitory potency of the ionophore on steroidogenesis of cells, but neither condition resulted in complete inhibition. Although greater inhibitory effects of monensin could be achieved under these

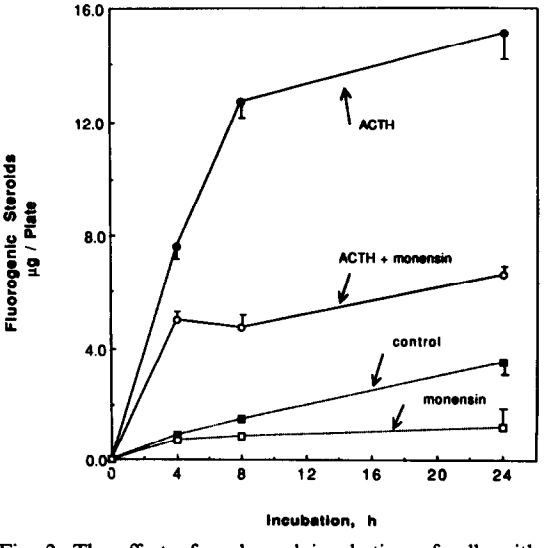

Fig. 2. The effect of prolonged incubation of cells with monensin on steroidogenesis. Cells were stimulated with ACTH (100 mU/ml) in presence or absence of monensin (1.2 μM) for 4, 8, and 24 h. Data are presented as means ± SD of 4 determinations.

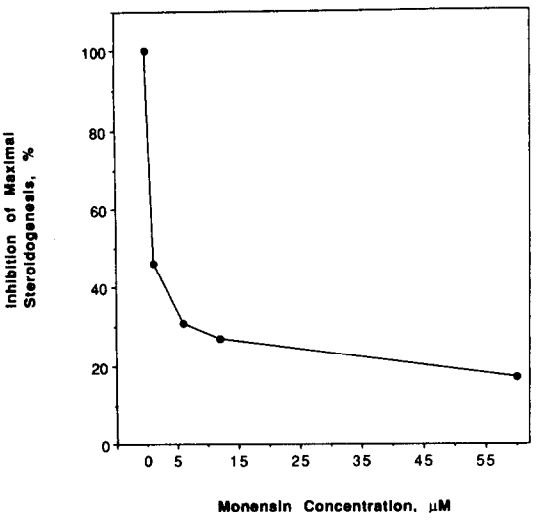

Fig. 3. The effect of strengthened monensin concentrations on steroidogenesis: cells were stimulated with ACTH (100 mU/ml) in the presence of 0 (control), 1.2, 6, 12, and 60 μM monensin for 4 h. Data are presented as % of the control.

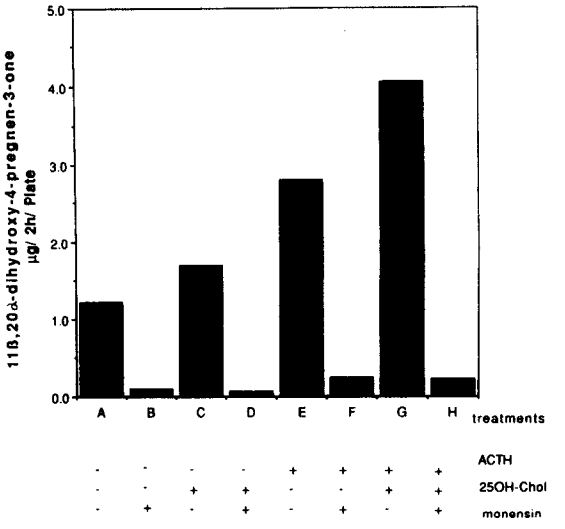

Fig. 4. The effect of 25-hydroxycholesterol on monensin-induced inhibition of steroidogenesis. Cells were preincubated with or without monensin (1.2 μM) for 30 min. After removal of ionophore-containing medium, cells were washed and then stimulated with ACTH (100 mU/ml) in the presence or absence of 25-hydroxycholesterol (15 μg/ml) in fresh and monensin-free medium for 2 h. The content of 11β,20α-dihydroxy-4-pregnen-3-one was determined by reversed phase HPLC using 17-hydroxyprogesterone as an internal standard.

conditions, the possibility of undesirable side-effects was also increased. For the following experiments, the concentration of monensin used was kept at 1.2 μM and the maximal time for exposure of cells to the ionophore was kept at 4 h or less, unless specified otherwise.

In order to label endogenously synthesized cholesterol, cells were preincubated with [^{14}C]acetate. As demonstrated in Table 1, the impairment of fluorogenic steroidogenesis of cells in the presence of monensin was accompanied by diminished incorporation of [^{14}C]acetate into the end-product steroid 11β,20α-dihydroxy-4-pregnen-3-one (Table 1). Because ^{3}H-labeled 11β,20α-dihydroxy-4-pregnen-3-one was not available for use as a recovery marker, the inhibitory effect on specific radioactivity in the steroid could not be precisely estimated. We subsequently developed a specific method to determine this steroid using reversed phase HPLC [16]; this method was used in the following experiment.

In order to investigate the possibility that insufficient delivery of cholesterol to mitochondria was the factor causing the impaired steroidogenesis of monensin-treated cells, the effect of 25-hydroxycholesterol on the steroidogenic activity was determined. This sterol had been known to be a readily active substrate for both rat luteal [19] and adrenal [20] steroidogenesis. If the impairment was indeed due to a limitation of cholesterol availability, supplementation of the cells with 25-hydroxycholesterol should be able to reverse the inhibitory effect. However, as determined by reversed phase HPLC, 25-hydroxycholesterol increased 11,β,20α-dihydroxy-4-pregnen-3-one synthesized by the cells but did not reverse the inhibitory potency of monensin on steroid production (Fig. 4).

Table 1. Monensin inhibits steroidogenesis in Y-1 adrenal cells prelabeled with [^{14}C]acetic acid

Treatment of cells in culture	Fluorogenic steroids (μg/4 h/plate)	Radioactivity in 11β,20α-dihydroxy-4-pregnen-3-one (cpm/4 h/plate)
Control	2.57 ± 0.18	1104 ± 234
ACTH (100 mU/ml)	7.10 ± 0.18	2403 ± 352
Monensin (1.2 μM)	1.41 ± 0.20†	545 ± 132††
ACTH + monensin	3.78 ± 0.29*	1039 ± 487**

Endogenously synthesized cholesterol was prelabeled with [^{14}C]acetic acid (1.3×10^6 cpm/plate) in serum-free MEM for 18 h prior to the experiment. Cells were then stimulated with ACTH in the presence or absence of monensin in fresh PBS-RH medium for 4 h. The results are presented as means ± SD of 4 determinations (†$P < 0.0005$ vs control; *$P < 0.0005$ vs ACTH; ††$P < 0.005$ vs control; **$P < 0.0025$ vs ACTH).

We subsequently used L-[^{35}S]methionine as a probe to investigate the possibility that monensin inhibits *de novo* protein synthesis in cells and mitochondria. As shown in Fig. 5, incubation of cells with monensin for 1–4 h resulted in a small but significant inhibition of the incorporation of L-[^{35}S]methionine into cell proteins. As shown in Table 2, the inhibition was greatly increased in mitochondria. The synthesis of proteins in mitochondria was inhibited approximately 40% by monensin (42 ± 13% inhibition summarized from three separate experiments under essentially similar conditions). In comparison, cycloheximide, an inhibitor of cytosolic ribosomal protein synthesis, inhibited protein synthesis by 80 ± 2%. Initial studies of mitochondrial protein autoradiograms by u.v. scanning revealed a complex pattern of proteins resulting from monensin treatment in Y-1 cells, suggesting more than one mitochondrial protein was affected.

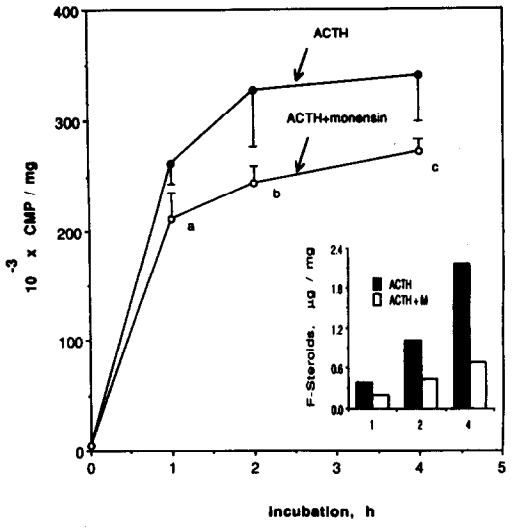

Fig. 5. The effect of monensin on incorporation of L-[^{35}S]methionine into cell homogenate proteins. Cells were stimulated with ACTH (100 mU/ml) in the presence of L-methionine (0,05 μg/ml) and L-[^{35}S]methionine (40 μCi/ml) with or without monensin (1.2 μM) for 1, 2, and 4 h. Aliquots of cell homogenate were taken to measure protein content and radioactivity. Data are presented as means $\pm$ SD from 8 determinations ($^{a}P < 0.0005$; $^{b}P < 0.001$; $^{c}P < 0.001$; vs ACTH at 1,2, and 4 h, respectively). The insertion presents the content of fluorogenic (F) steroids synthesized by the cells (M, monensin).

DISCUSSION

As indicated previously, treatment of Y-1 cells with monensin results in disruption of the organized structure of the Golgi complex and inhibition of both steroidogenesis of cells and cholesterol side-chain cleavage activity in isolated mitochondria [10]. Studies of monensin effects on cholesterol transport to mitochondria in Y-1 cells have technical limitations. Previous demonstration of ACTH-stimulated transport of cholesterol to rat adrenal mitochondria was achieved in intact rats [21] or rat adrenal cells [22] using aminoglutethimide. Because this inhibitor blocks the mitochondrial $P450_{scc}$ reaction without affecting cholesterol transport, the increased portion of cholesterol in the mitochondria compared to control would represent the maximal level of cholesterol transferred from outside the organelle during the incubation period. However, Hall *et al.*[23] and our laboratory have found that the cholesterol content of Y-1 mitochondria did not increase by this approach. Hall *et al.*[23] suggested that Y-1 mitochondria contained a large amount of cholesterol which caused the background to be too high hampering the measurement of a relatively small amount of cholesterol transfer. Nakamura *et al.*[24] reported that cholesterol accumulation was found in the Y-1 inner mitochondrial membrane after removal of the outer membrane. Nevertheless, failure of the attempt to reverse the inhibitory effect on steroidogenesis by supplementation of monensin-treated cells with 25-hydroxycholesterol (Fig. 4) suggests that cholesterol availability for steroidogenic reactions may not be the sole factor for the inhibition.

In contrast, our data (Table 2) suggests that the diminished incorporation of labeled methionine into newly synthesized proteins in mitochondria may be associated with the impaired mitochondrial cholesterol side-chain cleavage activity [10]. In this context, 1 μM monensin does not interfere with *de novo* protein synthesis in many other cells [25–27]. Yet, the present data indicates that *de novo* protein synthesis in Y-1 cells appears to be sensitive to the ionophore at this level. Because monensin reversibly inhibits steroidogenesis [11] and does not interfere with rounding up of cells in response to ACTH stimulation (unpublished observation), it is unlikely that the decreased capability is due to a toxic effect. It should be noted that frog retinal protein synthesis is inhibited at levels as low as 0.1 μM monensin [28]. Whether the decline of newly synthesized proteins in the mitochondria is a consequence of, or independent from, the impairment of *de novo* protein synthesis in whole cells (Fig. 5) remains to be elucidated.

The function of the Golgi complex in sorting proteins to plasma membrane and lysosomes has been well established (for a review see [29]). To our knowledge, there has been no indication that mitochondrial proteins are sorted by the Golgi complex. Most mitochondrial proteins are synthesized at free ribosomes [30], whereas the proteins sorted by the Golgi complex are synthesized at endoplasmic reticulum membrane-bound ribosomes. However, Nabi *et al.* [31, 32] previously reported that, in bovine adrenal cortex, the synthesis of nascent adrenodoxin, one of the protein components of mitochondrial *P*450 enzymes, is closely associated with the fraction of tightly membrane-bound endoplasmic reticulum ribosomes as well as relatively small fractions of free, and loose membrane-bound ribosomes. On the other hand, the synthesis of mitochondrial adrenodoxin reductase, $P450_{scc}$ and *P*45011β is predominantly

Table 2. The effect of monensin on L-[^{35}S]methionine incorporation into newly synthesized mitochondrial proteins

Treatment of cells in culture	Fluorogenic steroids synthesized by the cells (μg/4 h/plate)	Radioactivity in isolated mitochondrial proteins (10^{-3} x cpm/mg)
ACTH (100 mU/ml)	2.05 $\pm$ 0.17	3363 $\pm$ 815
ACTH + monensin (1.2 μM)	0.68 $\pm$ 0.03*	1886 $\pm$ 185***
ACTH + cycloheximide (50 μg/ml)	0.56 $\pm$ 0.07**	648 $\pm$ 57****

Cells were stimulated with ACTH in the medium containing L-methionine (0,05 μg/ml) and L-[^{35}S]methionine (40 μCi/ml) with or without monensin, or cycloheximide, for 4 h. Data are presented as means $\pm$ SD of 4–8 determinations (*$P < 0.0005$; **$P < 0.0005$; ***$P < 0.05$; ****$P < 0.0005$; vs ACTH).

associated with fractions of free and loosely membrane-bound ribosomes. The authors suggested that the synthesis of adrenodoxin peptide starts on free ribosomes, and the ribosomes attach to the membrane of endoplasmic reticulum before completion of adrenodoxin synthesis. We have previously demonstrated that the mitochondrial adrenodoxin level of Y-1 cells is stimulated by ACTH [13]. Whether the requirement of the membrane of the endoplasmic reticulum for completion of adrenodoxin synthesis is linked to the aformentioned activation of the GERL region by ACTH stimulation [7], and whether this synthesis is inhibited by monensin, are not known at present.

The GERL region and mitochondria in the adrenal cortex can be considered as highly specialized compartments. In this aspect, Jorgensen *et al.*[33] reported that, in the cultured rat skeletal muscle cells, calsequestrin, a sarcoplasmic reticulum calcium binding protein, is first transferred to the Golgi complex after being synthesized in endoplasmic reticulum, and then transferred to the sarcoplasmic reticulum. Thomas *et al.*[34] further demonstrated that the newly synthesized calsequestrin, destined for the sarcoplasmic reticulum, is contained in Golgi-derived clathrin-coated vesicles. Thus, the possibility that the Golgi complex regulates a highly specialized compartment other than plasma membrane and lysosomes exists. Indeed, we have previously shown an increased activity of vesicles either associated with or close to the hypertrophied Golgi complex of Y-1 cell stimulated with ACTH [4, 10]; this activity is diminished by monensin [10].

Import of proteins into the inner mitochondrial membrane in general is an energy-dependent and uncoupler-sensitive process (for a review see [35]). One would suggest that monensin might simply act as an uncoupler of mitochondria, which results in impairment of the import of the enzyme protein components and a resultant decline of enzymic function. Such an inhibitory mechanism could be independent from the Golgi disruption we observed [10, 11]. However, our studies indicated that 12–20 μM 2,4-dinitrophenol, a well-known mitochondrial uncoupler, inhibits steroidogenesis of Y-1 cells less than 10% ([11] and our unpublished observation). Indeed our earlier data have shown that to achieve a comparable extent of inhibition on steroidogenesis found with 1.2 μM monensin, 0.1–1 mM 2,4-dinitrophenol is required [13]. Byczkowski and Hac[36] previously reported that the I_{50} of 2,4-dinitrophenol for suppression of rat liver mitochondrial bioenergetic function is approximately 9 μM. Thus, only a small fraction of the inhibitory effect of monensin on steroidogenesis of cells could be attributed to an uncoupling reaction at the mitochondria.

Based upon the above considerations, we speculate that, in ACTH-stimulated Y-1 adrenal cells, there is a monensin-sensitive mechanism in the regulation of mitochondrial *de novo* protein synthesis, and the Golgi complex or its related compartment(s) may be involved in this regulation. To further define the types of mitochondrial proteins impaired by monensin action requires studies at submitochondrial as well as extramitochondrial levels using specific mitochondrial proteins and enzymes as markers. In parallel, characterization of the isolated Golgi complex from the cells might also provide clues for the elucidation of its role.

Acknowledgements—We wish to thank Professors David Chuang and Rody Cox for the unlimited use of their HPLC facility in the earlier phase of this work. The general laboratory assistance provided by Anthony Honarvar and James Chung (summer student assistant) are also grateful. This work was supported by the Veterans Administration Medical Service.

REFERENCES

1. Reese J. D. and Moon H. D.: The Golgi apparatus of the cells of the adrenal cortex after hypophysectomy and on the administration of the adrenocorticotropic hormone. *Anat. Rec.* **70** (1938) 543–556.
2. Rebuffat P., Cavallini L., Belloni A. S., Mazzocchi G., Coi A., De Tos G. P. and Nussdorfer G. G.: A morphometric study of the reversal of ACTH-induced hypertrophy of rat adrenocortical cells after cessation of treatment. *J. Submicrosc. Cytol. Path.* **21** (1989) 73–81.
3. Kahri A. I.: Inhibition by cycloheximide of ACTH-induced internal differentiation of mitochondria in cortical cells in tissue culture of fetal rat adrenals. *Anat. Rec.* **171** (1971) 53–79.
4. Mattson P. and Kowal J.: Acute steroidogenic stimulation of cultured adrenocortical tumour cells: an elctron microscopic analysis. *Tiss. Cell Res.* **12** (1980) 685–701.
5. Magalhaes M. C. and Magalhaes M. M.: Ultrastructural alterations produced in rat adrenal by aminoglutethimide; a stereologic and cytochemical study. *Endocrinology* **90** (1972) 444–452.
6. Magalhaes M. C. and Magalhaes M. M.: Effects of alpha-amanitin on the fine structure of adrenal fasciculata cells in the young rat. *Tissue Cell* **17** (1985) 27–37.
7. Kowal J., Mattson P. and Cheng B.: Intracellular events associated with ACTH action. *Endocr. Res.* **10** (1984–85) 463–472.
8. Nossdorfer G. G., Mazzocchi G. and Meneghelli V.: Cytophysiology of the adrenal zona fasciculata. *Int. Rev. Cytol.* **55** (1978) 291–356.
9. Tartakoff A.: Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32** (1983) 1026–1028.
10. Cheng B., Horst I. A. and Kowal J.: The Golgi apparatus and adrenal function: the effects of monensin on adrenocorticotropic hormone-stimulated steroidogenesis. *Arch. Biochem. Biophys.* **239** (1985) 508–516.
11. Cheng B., Horst I. A. and Kowal J.: The effects of monensin on inhibition of steroidogenesis and disruption of the Golgi complex in adrenal cells are both reversible. *Endocr. Res.* **13** (1987) 243–250.
12. Mastsuka H, Ishii M., Yamakado M. and Uehara Y.: Effect of natrium ionophore on aldosterone production in the rat adrenal gland. *Endocrinology* **115** (1984) 2039–2044.
13. Kowal J.: ACTH and the metabolism of adrenal cell cultures. *Recent Prog. Horm. Res.* **26** (1970) 623–687.
14. Kowal J., Frenkel R. and Angee I.: Adrenal cell in culture. IX: the site of ACTH stimulation of glucose metabolism. *Endocrinology* **91** (1972) 1219–1226.

15. Kowal J. and Fiedler R.: Adrenal cells in tissue culture I. assay of steroid products; steroidogenic response to peptide hormones. *Arch. Biochem. Biophys.* **128** (1968) 406–421.
16. Cheng B. and Kowal J.: A specific reversed phase high performance liquid chromatographic method for analysis of steroids in Y-1 adrenal cell cultures. *J. Chromat.* **432** (1988) 302–307.
17. Ray D. B., Horst I. A. and Kowal. J.: Adrenocorticotropic hormone increases specific proteins of the mitochondrial fraction that are translated inside or outside this organelle in cultured adrenal tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* **77** (1980) 4648–4652.
18. Bensadoum A. and Weinsten D.: Assay of proteins in the presence of interfering materials. *Analyt. Biochem.* **70** (1976) 241–250.
19. Toaff M. E., Schleyer H. and Strauss III J. F.: Metabolism of 25-hydroxycholesterol by rat luteal mitochondria and dispersed cells. *Endocrinology* **111** (1982) 1785–1790.
20. Popplewell P. Y. and Azhar S.: Effects of aging on cholesterol content and cholesterol-metabolizing enzymes in the rat adrenal gland. *Endocrinology* **121** (1987) 64–73.
21. Mahaffee D., Reitz R. C. and Ney R. L.: The mechanism of action of adrenocorticotropic hormone; the role of mitochondrial cholesterol accumulation in the regulation of steroidogenesis. *J. Biol. Chem.* **249** (1974) 227–233.
22. Privalle C. T., McNamara B. C., Dhariwal M. S. and Jefcoat C. R.: ACTH control of cholesterol side-chain cleavage at adrenal mitochondrial cytochrome P450scc; regulation of intramitochondrial cholesterol transfer. *Molec. Cell. Endocr.* **53** (1987) 87–101.
23. Hall P. F., Charpponnier C., Nakamura M. and Gabbiani G.: The role of microfilaments in the response of adrenal tumor cells to adrenocorticotropic hormone. *J. Biol. Chem.* **254** (1979) 9080–9084.
24. Nakamura M., Watanuki M., Tilley B. E. and Hall P. F.: Effect of adrenocorticotrophin on intracellular cholesterol transport. *J. Endocr.* **84** (1980) 179–188.
25. Tartakoff A. M. and Vassalli P.: Plasma cell immunoglobulin secretion arrest is accompanied by alterations of the Golgi complex. *J. Exp. Med.* **146** (1977) 12332–12345.
26. Uchida N., Smilowitz H. and Tanzer M.: Monovalent ionophore inhibits secretion of procollagen and fibronectin from cultured human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **76** (1979) 1868–1872.
27. Marnell M. H., Stookey M. and Draper R. K.: Monensin blocks the transport of diphtheria toxin to the cell cytoplasm. *J. Cell. Biol.* **93** (1982) 57–62.
28. Fliesler S. J. and Basinger S. F.: Monensin stimulates glycerolipid incorporation into rod outer segment membranes. *J. Biol. Chem.* **262** (1987) 17516–17523.
29. Rothman J. E.: Protein sorting by selective retention in the endoplasmic reticulum and Golgi stack. *Cell* **50** (1987) 521–522.
30. Suissa M. and Schatz G.: Import of proteins into mitochondria translatable mRNAs for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes. *J. Biol. Chem.* **257** (1982) 13048–13055.
31. Nabi N., Ishikawa T., Ohashi M. and Omura T.: Contribution of cytoplasmic free and membrane-bound ribosomes to the synthesis of NADPH-adrenodoxin reductase and adrenodoxin of bovine adrenal cortex mitochondria. *J. Biochem.* **94** (1983) 1505–1515.
32. Nabi N., Kominami S., Takemori S. and Omura T.: Contribution of cytoplasmic free and membrane-bound ribosomes to the synthesis of mitochondrial cytochrome P-450(SCC) and P-450 (11β) and microsomal P-450 (C-21) in bovine adrenal cortex mitochondria. *J. Biochem.* **94** (1983) 1517–1527.
33. Jorgensen A. O., Kalnins V. I., Zubrzycka E. and MacLennan D. H.: Assembly of the sarcoplasmic reticulum localization by immunofluorescence of sarcoplasmic reticulum proteins in differentiating rat skeletal muscle cell culture. *J. Cell. Biol.* **74** (1977) 287–298.
34. Thomas K., Navarro J., Benson R. J. J., Cambell K. P., Rotundo R. L. and Fine R. E.: Newly synthesized calsequestrian, destined for the sarcoplasmic reticulum, is contained in early/intermediate Golgi-drived clathrin-coated vesicles. *J. Biol. Chem.* **264** (1989) 3140–3145.
35. Pfanner N., Hartl F.-U. and Neupert W.: Import proteins into mitochondria: a multiple-step process. *Eur. J. Biochem.* **175** (1988) 205–212.
36. Byczkowski J. Z. and Hac E. E.: Relationship between inhibition of prostaglandin β-oxidation and deenergization of mitochondrial membranes. *Acta Biol. Med. Germ.* **37** (1978) 895–896.