STIMULATION OF UPTAKE OF CHOLESTERYL ESTERS INTO ADRENAL TUMOR CELLS BY ACTH AND OTHER AGENTS

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1. Introduction

It is well known that ACTH causes a rapid (< 5 min) increase in the production of corticosteroids by adrenocortical cells, both normal [1] and malignant [2]. This response is believed to result from increased conversion of cholesterol to pregnenolone in mitochondria [1–4]. This acute response is seen without the addition of exogenous substrate for steroid synthesis and is inhibited by cytochalasin B [5,6] and by anti-actin (to be published). Studies in vivo show that the major source of adrenal cholesterol for steroid synthesis comes from the circulating plasma [7,8]. Moreover, there is reason to believe that this substrate enters adrenal cells in the form of esterified cholesterol [9]. We have observed that ACTH does not influence transport of cholesterol into Y-1 adrenal tumor cells [6]. It is important to determine whether ACTH and other agents capable of stimulating steroidogenesis, influence the transport of cholesterol ester into adrenal cells. For this purpose, we have used Y-1 adrenal tumor cells and report here that ACTH, dibutyryl cyclic AMP and certain other agents accelerate the transport of cholesterol esters into these cells.

2. Materials and methods

The relevant methods for incubating Y-1 cells measuring steroid (20α-dihydroprogesterone) production and measuring transport of cholesterol and other steroids into these cells have been given in [5,6].

[^{3}H]Cholestryl linoleate was prepared using the procedure in [9]. For the final purification, thin-layer chromatography on silica gel in heptane/diethyl ether/acetate (85:15:2) [10] was used instead of column chromatography on silicic acid/celite. The purity of the final product was 98% as determined by thin-layer chromatography.

[1,2-^{3}H]Cholesterol (43 Ci/mmol), linoleoyl chloride and cholesteryl linoleate were purchased from New England Nuclear Corp. Supelco. and Applied Science Lab., respectively. Other chemicals were obtained from sources described in [5,6].

Uptake of cholesteryl linoleate was measured by incubating cells with [^{3}H]cholesteryl linoleate (100 000 cpm; 1.05 pmol/dish). Half of the solution of [^{3}H]cholesteryl linoleate was diluted to 100 000 cpm/1.0 nmol and cells were incubated in pairs: one dish with the standard solution; a second with the diluted solution. Specific uptake was determined by subtraction [11]. At 60 min, non-specific uptake was ~60% of total. Values for specific uptake were negligible at 0°C and at t = O.

Cells were washed with phosphate-buffered saline and incubated in minimal Eagle's medium (no serum) containing ACTH or 0.01% (w/v) bovine serum albumin or other additions as indicated. [^{3}H]Cholesterol ester (10 000 cpm; 0.2 pmol/ml) or [^{3}H]cholesterol was added in *N,N*-dimethylformanide (50 μl). Following incubation, cells were washed twice with phosphate buffered saline; the second wash contained < 2% of the radioactivity remaining in the cells. The cells were then scraped from the plates in 4 ml buffered saline and the cell suspension was extracted 3 times with diethyl ester. The combined extracts were dried and applied to thin-layer chromatograms

in heptane : diethyl ether : acetic acid (85:15:2, v/v/v). The [^{3}H]cholesterol ester was measured by liquid scintillation spectrometry. Each plate was accompanied by a comparison plate which was treated in an identical manner except that 100-fold excess of unlabeled cholesterol ester (20 pmol) was added with the ^{3}H-labeled ester. Specific binding was determined by difference [11]. Unless otherwise stated, the data which follow show total binding.

A number of other solvents were used to dissolve the cholesterol esters, e.g., Tween-80, propylene glycol, dioxane. These solvents did not appreciably alter the results obtained. The following peptide hormones were used as control substances: LH, ovine NIH LH; insulin, bovine Eli Lilly; vasopressin, synthetic lysine vasopressin Sigma Chemical Co.; TSH, ovine NIH; glucagon, crystalline glucagon Eli Lilly.

3. Results

Figure 1 shows that ACTH increases the transport of [^{3}H]cholesteryl linoleate into Y-1 adrenal cells.

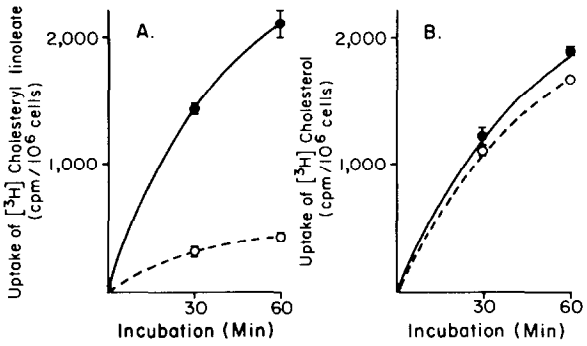

Fig.1. The influence of ACTH on the transport of [^{3}H]cholesterol linoleate and [^{3}H]cholesterol into Y-1 cells. Y-1 cells were incubated in minimal Eagle's medium with [2,3-^{3}H]cholesteryl linoleate (100 000 cpm, 1.05 pmol/dish) (A) or with [2,3-^{3}H]cholesterol (100 000 cpm, 1.05 pmol/dish) (B) and either ACTH (86 mU/ml) or bovine serum albumin (0.01%) (control). At the times shown, plates were washed twice with phosphate-buffered saline and extracted with ether. Extracts were dried and applied to thin-layer plates to separate free and esterified cholesterol [10] which were measured by liquid scintillation spectrometry. Values shown are means and ranges for triplicate determinations. Open symbols: control. Closed symbols: ACTH.

Transport of [^{3}H]cholesterol was not significantly increased. Stimulation by ACTH to approximately the same extent as that seen with cholesteryl linoleate was observed when cholesteryl oleate was used (data not shown). Similar results were obtained when steroid synthesis was inhibited by aminoglutethimide (7.6 mM) because conversion to steroids is small relative to cholesterol uptake at < 1 h incubation [6].

Table 1 shows that dibutyryl cyclic AMP stimulates the specific uptake of cholesteryl esters (linoleate and oleate) in Y-1 cells and that ACTH also stimulates uptake of the oleate ester. ACTH was without effect on uptake of 5α-androstanediol-3β-linoleate and that of 5α-dihydrotestosterone-17β-linoleate. Table 1 also shows that the action of ACTH was not inhibited by a concentration of aminoglutethimide that is known completely to inhibit steroidogenesis [6].

Figure 2 shows that concanavalin A stimulates transport of [^{3}H]cholesteryl linoleate into Y-1 cells without increasing the production of 20α-dihydroprogesterone (a major product of steroidogenesis in Y-1 cells). Figure 3 shows that cytochalasin B also stimulates transport of cholesteryl linoleate into Y-1 cells. These studies were performed in medium not containing serum [6,7]. When cells were incubated with serum and [^{3}H]cholesteryl linoleate, cytochalasin B still stimulated transport of this ester but to no greater extent than without serum, e.g., control: 846 ± 31; cytochalasin B (5×10^{-6} M) : 1949 ± 91; control in serum 648 ± 18; and cytochalasin B (5×10^{-6} M) in serum 1448 ± 97; (cpm/h/10^6 cells); values given are means and ranges for triplicate determinations. Finally, cytochalasin B did not influence the response to ACTH whether the concentration of ACTH used provided maximal or submaximal stimulation (data not shown).

Table 2 shows that much of the total radioactivity (>45%, data not shown) associated with the whole cells after incubation with cholesteryl linoleate, is found in mitochondrial and supernatant fractions; evidently the ester enters the cells. When cells were incubated at 2°C, <5% of total radioactivity was found in these fractions. In 3 experiments, it was observed that differences seen in both whole homogenate and mitochondria are statistically significant ($p<0.01$). Table 3 shows that a number of peptide hormones other than ACTH are without effect on uptake of cholesteryl linoleate.

Table 1
The influence of ACTH and cyclic AMP on uptake of cholesteryl esters by Y-1 cells

Substrate	Addition	Specific uptake of ester (cpm/h/10^6 cells)
[^{3}H]Cholesteryl-linoleate	–	216 ± 23
	db[a] C'AMP (10^{-5} M)	511 ± 18
	db C'AMP (10^{-4} M)	694 ± 39
	db C'AMP (10^{-3} M)	787 ± 31
[^{3}H]Cholesteryl-oleate	–	387 ± 41
	ACTH (86 nmol/ml)	641 ± 37
	db C'AMP (10^{-5} M)	724 ± 29
	db C'AMP (10^{-4} M)	917 ± 31
	ACTH (86 nmol/ml) + Aminoglutethimide (0.76 mM)	660 ± 29
[^{3}H]Androstanediol-3β-linoleate	–	< 50
	ACTH 86 (nmol/ml)	< 50
5α-DHT[b]-linoleate	–	124 ± 13
	ACTH 86 (nmol/ml)	117 ± 18

[a] db, dibutyryl; [b]DHT, 5α-dihydrotestosterone

Specific uptake of esters was determined as in section 2 using conditions in the legend to fig.1. The values are means and ranges for triplicate determinations at 60 min. When aminoglutethimide was used, cells were pre-incubated with this substance (0.76 mM) for 15 min to inhibit steroidogenesis; subsequent solutions contained the inhibitor at the same concentration

Table 2
The distribution of intracellular [^{3}H]cholesteryl linoleate in Y-1 cells

Cell fraction		[^{3}H]cholesteryl oleate (cpm/mg protein)
Whole homogenate	1295 ± 90	1680 ± 350
Cell debris	1300 ± 400	1150 ± 400
Mitochondria	3460 ± 500	4140 ± 400
Post-mitochondrial supernate	200 ± 30	215 ± 20

Experimental conditions are in the legend to fig.1. An aliquot of the whole homogenate was examined before cell fractionation. Cell fractions were prepared as in [5,6]. The total recovery of radioactivity in the various fractions was 80–90% of the total radioactivity in the whole homogenate. Values shown are means and ranges for duplicate determinations

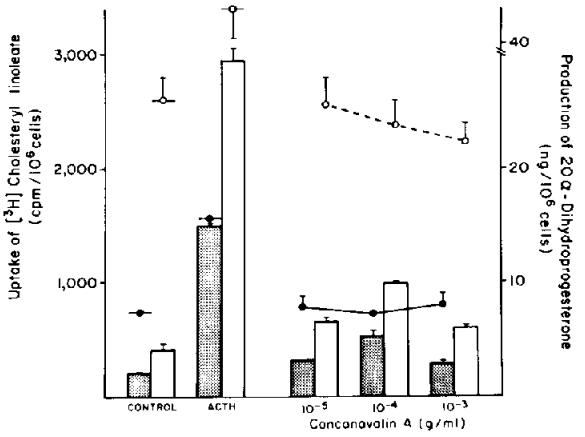

Fig.2. The influence of concanavalin A on the transport of cholesteryl linoleate into Y-1 cells. Cells were incubated with [2,3-^{3}H]cholesterol linoleate for 1 h and 3 h and transport of the labeled substrate was measured. For conditions, see legend to fig.1. Production of 20α-dihydroprogesterone was measured in the same samples [5,6]. Closed symbols: 1 h. Open symbols: 3 h. Bars represent uptake of [^{3}H]cholesteryl linoleate. Circles represent production of 20α-dihydroprogesterone.

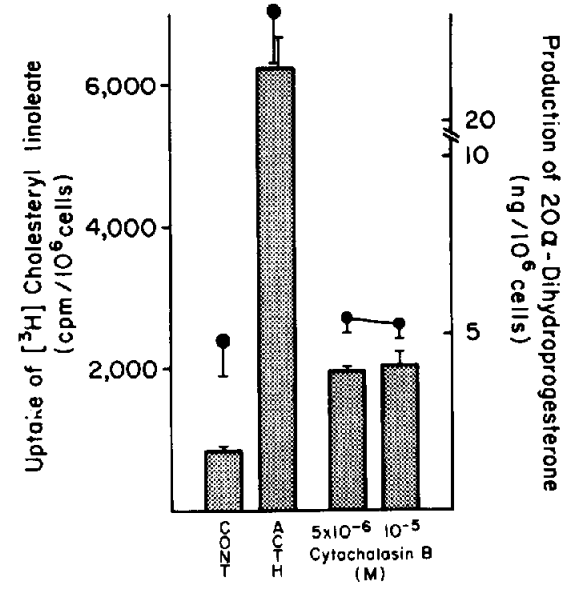

Fig.3. The influence of cytochalasin B on the transport of cholesteryl linoleate into Y-1 adrenal cells. Experimental details are given in legends to fig.1,2. Cells were incubated with or without cytochalasin B for 1 h. Bars represent uptake of [^{3}H]cholesteryl linoleate. Circles represent production of 20α-dihydroprogesterone. CONT: control.

Table 3
The influence of various peptides on transport of [^{3}H]cholesteryl linoleate into Y-1 cells

	[^{3}H]cholesteryl linoleate uptake (cpm/10^6 cells)
Control	1320 ± 75
ACTH (86 mU/ml)	1784 ± 112
LH (50 μg/ml)	1190 ± 131
Insulin (100 mU/ml)	1274 ± 64
Vasopressin (25 μg/ml)	1332 ± 74
TSH (50 μg/ml)	1302 ± 24
Glucagon (50 μg/ml)	1361 ± 17

Experimental details are in the legend to fig.1. Values are means and ranges of triplicate determinations

4. Discussion

These studies reveal a new effect of ACTH in vitro. Previous studies have demonstrated an effect of ACTH in vivo upon transport of cholesterol into adrenal cells when serum or lipoproteins were present [12,13]. The experiments reported here show that ACTH added to Y-1 cells in serum-free medium promotes uptake of cholesterol ester into Y-1 cells. In spite of the high non-specific binding of such compounds to cells, clear stimulation was observed when non-specific binding was subtracted. Moreover, dibutyryl cyclic AMP also stimulates transport of cholesterol esters in these cells. Concanavalin A stimulates transport of cholesterol ester without stimulating steroid synthesis under the condition we used; presumably the lectin influences the properties of the cell membrane. Cytochalasin B exerts a similar effect which is of interest in view of a recent report showing that this substance stimulates steroidogenesis by Y-1 cells only in the presence of serum [14]. Our results show that the effect of cytochalasin B on the transport process does not require serum; presumably serum provides cholesteryl ester which is transported into the cells at a greater rate under the influence of cytochalasin B. Whether or not such transport has the effect of enhancing steroidogenesis may depend on the state of the cells, e.g., serum may influence the utilization of cholesterol for growth and other processes which could compete with steroid synthesis for this substance. Under such conditions, increased cholesterol

transport could increase steroidogenesis. Such stimulation of steroid synthesis is not seen without serum (our results and [14]). The important point is that the two effects can be separated: ACTH, cyclic AMP and other agents stimulate transport of cholesterol esters; ACTH and cyclic AMP can stimulate steroidogenesis from endogenous precursors (where transport of substrate is not involved); cytochalasin B and concanavalin A stimulate transport of cholesterol ester under conditions where steroidogenesis is not enhanced. Moreover, the effect of ACTH on uptake of cholesterol ester was not inhibited when steroidogenesis was inhibited by aminoglutethimide; evidently the response is not secondary to increased utilization of substrate for steroidogenesis.

Clearly ACTH exerts at least two distinct effects both of which may require cyclic AMP:

1. An effect on the transport of what may be the physiological substrate (cholesterol ester);
2. An intracellular effect on steroidogenesis which requires microfilaments, since it is inhibited by cytochalasin B and anti-actin (see section 1).

The first effect of ACTH is shared by a variety of agents which exert non-specific effects on the cell membrane. The second effect is shared by cyclic AMP. Cytochalasin B presumably stimulates steroidogenesis by way of the first effect since it inhibits the second action of ACTH and cyclic AMP (increased steroidogenesis from endogenous precursors) by inhibiting the action of microfilaments [5,6].

Acknowledgements

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