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Inhibition of Replication in Functional Mouse Adrenal Tumor Cells by Adrenocorticotropic Hormone Mediated by Adenosine 3′:5′-Cyclic Monophosphate

(steroidogenesis/morphological change/RNA and protein synthesis)

HIDEO MASUI AND LEONARD D. GARREN*

Division of Endocrinology, Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, Calif. 92037

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ABSTRACT **Adrenocorticotropic hormone (ACTH) inhibited replication in functional adrenal tumor cells with a concomitant stimulation of steroidogenesis and a characteristic change of morphology from a flattened to a spherical type. [^{3}H]Thymidine incorporation into DNA was inhibited by about 50% 6 hours after ACTH treatment. Both cyclic AMP and dibutyryl cyclic AMP inhibited [^{3}H]thymidine incorporation and caused the characteristic morphological change noted with ACTH. The extent of stimulation of steroidogenesis and the amount of inhibition of [^{3}H]thymidine incorporation in response to various doses of ACTH were closely related and both were in parallel with the concentration of cyclic AMP in the cells. Cyclic GMP and cyclic IMP did not inhibit [^{3}H]thymidine incorporation significantly, and did not change the morphology of the cells. AMP inhibited [^{3}H]thymidine incorporation into DNA and caused the characteristic morphological change. However, AMP did not increase the cyclic AMP content of the cells. CMP, GMP, and UMP showed a significant inhibition of [^{3}H]thymidine incorporation into DNA, but the extent of the inhibition was much less than that with AMP. These nucleotides did not change the morphology of the cells.**

Adenosine 3′:5′-cyclic monophosphate (cAMP) mediates intracellularly the physiological functions of various hormones in animal tissues (1). In the adrenal cortex, ACTH activates adenylate cyclase on the plasma membrane of the cells, resulting in an increased concentration of cAMP that mediates the intracellular functions of ACTH (steroidogenesis and synthesis of macromolecules in the adrenal cortex (2, 3).

Gericke and Chandra observed that the administration of cAMP inhibits the growth of transplanted lymphosarcoma in mice (4). In several cultured tumor cell lines, an inhibitory effect of cAMP on the growth of the cells has been reported (5–7). Johnson *et al.* have shown that in chemically transformed fibroblasts (L-cells) and Rous sarcoma virus-transformed fibroblasts (XC-cells), dibutyryl adenosine 3′:5′-cyclic monophosphate (But_2cAMP) restores the morphological and growth characteristics of normal fibroblasts. No response to But_2cAMP was observed in various transformed epithelial cells (8). A similar phenomenon was reported by Sheppard with 3T6 cells (spontaneously transformed) and polyoma virus-transformed PyV-3T3 cells (9). Hsie *et al.* have found that in Chinese hamster ovary cells, But_2cAMP restores the contact-inhibition and morphology of normal cells and stimulates collagen synthesis in these fibroblasts (10, 11). Polyoma virus-transformed fibroblasts contain less adenylate cyclase activity than do untransformed fibroblasts (12). These observations suggest that cAMP may play an important role in the regulation of the growth of these cells, in addition to the well-documented role as the intracellular mediator of hormonal actions. In certain tumor cells, cAMP also tends to restore the characteristics of normal cells.

Functional mouse adrenal tumor cells were originally established by Sato *et al.* (13) and grown in tissue culture in the absence of ACTH. The most characteristic property of these tumor cells is that they retain the specific function of the adrenal cortex. ACTH activates adenylate cyclase of the plasma membrane of the cells (14), and both ACTH and cAMP stimulate steroidogenesis (15). This is a unique system in which to study the relationship between the control mechanisms of a specific function of these cells and of their growth and the role of cAMP in these control mechanisms. The present studies demonstrate that ACTH inhibits DNA synthesis in these cells, and that the inhibition of DNA synthesis seems to be mediated by cAMP.

MATERIALS AND METHODS

Cells. Functional mouse adrenal tumor cells were a gift from Dr. Gordon Sato (13). The medium used to grow the cells was F-10 mixture supplemented with 12.5% horse serum and 2.5% fetal-calf serum (Grand Island Biologicals). The cells were grown in plastic tissue-culture bottles (25 cm^2; 30 ml, Falcon Plastics) and incubated at 37°C in an atmosphere containing 5% CO_2. Experiments were performed 1–2 days after subculture, and each bottle contained about 1×10^6 cells and 3.0 ml of medium.

[^{3}H]Thymidine Incorporation into DNA and Determination of DNA. Unless otherwise specified, cells were incubated under experimental conditions for 6 hr. 2 Hr before the end of incubation, 10 μCi of [^{3}H]thymidine (6.0 Ci per mol, Schwarz/Mann) was added to each bottle. Then the medium was removed and DNA was isolated by the method described by Marmur (16). Aliquots of DNA extracts were precipitated with 1.0 N HCl. The precipitate was collected on glass-fiber discs and washed. The radioactivity in acid-insoluble material was measured in a liquid scintillation counter. DNA was measured by the method described by LePecq and Paoletti (17).

Abbreviations: ACTH, adrenocorticotropic hormone; $(But)_2$-cAMP, N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; cIMP, inosine 3′:5′-cyclic monophosphate.

* Dr. Leonard D. Garren, deceased, October 31, 1971.

TABLE 1. *Effect of ACTH on DNA content and number of functional adrenal tumor cells*

Incubation period (hr)	Treatment	DNA content (μg/bottle)	DNA content (% increase)	Cell number (per bottle × 10^{-6})	Cell number (% increase)
0		10.5 ± 2.8*		1.08 ± 0.06	
24	None	21.8 ± 2.2	108	2.43 ± 0.22	125
24	+ACTH	14.0 ± 1.0	33	1.38 ± 0.06	28

Cells were incubated with ACTH (0.3 unit/ml of medium) for 24 hr. Each group consisted of four cultures.
* Standard deviation.

Steroid Assay. Corticosteroids in the culture medium were extracted with methylene chloride and assayed according to the fluorescent method described by Silber *et al.* (18).

cAMP Assay. Cells from 5–6 bottles were combined to determine the cAMP content. The medium was removed, 1 ml of cold trichloroacetic acid was added to each bottle, and cells were collected and homogenized. The amount of cAMP was determined by the method described by Walton and Garren (19), except that cAMP was not purified on a Dowex column before assay.

RESULTS

Effect of ACTH on steroidogenesis and DNA synthesis

ACTH stimulated steroidogenesis in functional adrenal tumor cells and caused a characteristic change of the morphology of these cells from a flattened to a spherical form, as originally reported by Yasumura *et al.* (20). As shown in Fig. 1, [^{3}H]thymidine incorporation into DNA of these cells decreased rapidly during the first 6 hr of ACTH treatment. The incorporation was inhibited by more than 90% in 14 hr and remained low for another 12 hr. In control experiments, in which cells were incubated in the absence of ACTH, [^{3}H]thymidine incorporation did not change for 14 hr, but after 24 hr decreased by about 30%. The amount of corticosteroids in the medium increased proportionally with duration of ACTH treatment up to 26 hr. This demonstrates that a relatively constant steroidogenesis continued during this period, even after DNA synthesis was almost completely inhibited in 14 hr.

Since ACTH inhibited [^{3}H]thymidine incorporation into DNA of functional adrenal tumor cells, the effect of ACTH on the DNA content and cell number was also studied. Cells were grown for 24 hr in the presence and absence of ACTH and the DNA content and cell number were compared. As shown in Table 1, both the DNA content and cell number increased by about 100% in the absence of ACTH. In the presence of ACTH, both values increased only by about 30%, which demonstrates that ACTH inhibits both DNA synthesis and cell division in these cells. The finding that the increase in DNA content and cell number was only about 30% in the presence of ACTH, compared to the increase of about 100% in the control cells, is consistent with the results in which [^{3}H]thymidine incorporation showed a rapid decrease during the first 6 hr of ACTH treatment (Fig. 1). This result indicates that [^{3}H]thymidine incorporation reflects the rate of DNA synthesis under the present experimental conditions. To test whether the inhibition of DNA synthesis by ACTH is specific to cells derived from the adrenal cortex, human fibroblasts were incubated with ACTH (0.3 unit/ml) for 6 hr and [^{3}H]thymidine incorporation was measured. ACTH had no effect on [^{3}H]thymidine incorporation into DNA of human fibroblasts.

Effect of ACTH on RNA and protein synthesis

Since ACTH inhibits DNA synthesis of functional adrenal tumor cells, the effect of ACTH on RNA and protein synthesis was studied. The cells were grown for 6 hr with ACTH (0.3 unit/ml) and the incorporation of radioactive uridine or leucine into acid-insoluble material was measured. For comparison, [^{3}H]thymidine incorporation into DNA was assayed in parallel experiments. As shown in Table 2, [^{3}H]uridine incorporation was not changed significantly with ACTH treatment and [^{3}H]leucine incorporation was slightly increased, whereas [^{3}H]thymidine incorporation was markedly inhibited. This suggests that RNA and protein synthesis were not changed markedly by ACTH treatment during this period.

Effect of corticosteroid on DNA synthesis

It has been shown that corticosteroids inhibit replication of certain cells (21). Because functional adrenal tumor cells produce corticosteroids in response to ACTH, the inhibition of DNA synthesis in these cells might be due to the corticosteroids produced by the cells. To test this possibility, cortisone, hydrocortisone, or prednisone was added to the culture medium and the effect on [^{3}H]thymidine incorporation was investigated. As shown in Table 3, these corticosteroids at a concentration of 10 μg/ml of medium did not inhibit the incorporation significantly. After incubation for the same period, the corticosteroid concentration in the medium was about 0.4 μg/ml and, therefore, any effect on [^{3}H]thymidine

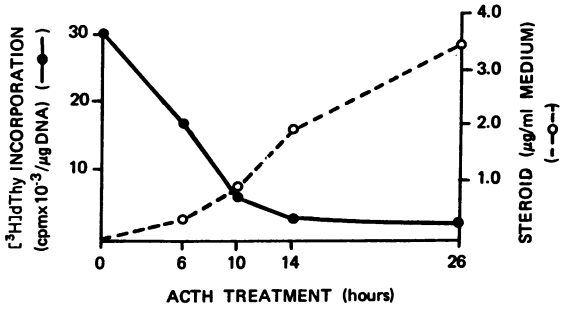

FIG. 1. Effect of ACTH on steroidogenesis and [^{3}H]thymidine incorporation into DNA of functional adrenal tumor cells. Cells were incubated with ACTH (0.3 unit/ml of serum) for the indicated periods of time. 2 Hr before the end of incubation, 10 μCi of [^{3}H]thymidine was added. [^{3}H]Thymidine incorporation into DNA and the amount of corticosteroids in the medium were determined. Each point represents the average of the values from three cultures.

TABLE 2. *Effect of ACTH on RNA and protein synthesis in functional adrenal tumor cells*

Treatment	[^{3}H]Uridine incorporation (cpm/bottle)	[^{3}H]Leucine incorporation (cpm/bottle $\times 10^{-3}$)	[^{3}H]Thymidine incorporation (cpm/bottle $\times 10^{-3}$)
None	2640 ± 280	91.2 ± 26.9	595 ± 76
ACTH	2340 ± 380	120.1 ± 27.4	107 ± 12
% change	−10	+32	−82

Cells were incubated with ACTH (0.3 unit/ml) for 6 hr. 2 Hr before the end of each incubation, 10 μCi of [^{3}H]uridine, [^{3}H]-leucine, or [^{3}H]thymidine was added. The incorporation of the radioactive uridine and leucine into acid-insoluble material was measured and [^{3}H]thymidine incorporation into DNA was assayed. Each group of the radioactive uridine, leucine, and thymidine incorporation experiments consisted of five, five, and four cultures, respectively.

incorporation would have been negligible. The time curve of the inhibition of [^{3}H]thymidine by ACTH, which showed an early rapid decrease after the addition of ACTH with little accumulation of corticosteroids in the medium (Fig. 1), also suggests that the inhibition of DNA synthesis is not due to accumulation of corticosteroids.

The possible inhibition of DNA synthesis by corticosteroids was also tested by the use of an inhibitor of steroidogenesis, aminoglutethimide, which blocks the conversion from cholesterol to Δ^5-pregnenolone (22). If the inhibition of DNA synthesis by ACTH is due to the accumulation of corticosteroids in the cells, ACTH would not be expected to inhibit DNA synthesis in the presence of an inhibitor of steroidogenesis. As shown in Table 4, 10 μg/ml of aminoglutethimide blocked the stimulation of steroidogenesis by ACTH. However, in the presence of aminoglutethimide, ACTH still inhibited [^{3}H]thymidine incorporation into DNA. 10 μg/ml of aminoglutethimide, itself, only slightly inhibited [^{3}H]-thymidine incorporation.

Effect of cAMP and other nucleotides on [^{3}H]thymidine incorporation into DNA

Since adenylate cyclase in these cells is stimulated by ACTH (14), the effect of cAMP on [^{3}H]thymidine incorporation into DNA of the functional adrenal tumor cells was studied. As shown in Table 5, at the concentration of 1 mM, both cAMP and But$_2$cAMP significantly inhibited [^{3}H]thymidine incorporation. The extent of the inhibition was less with cAMP than with But$_2$cAMP. Other cyclic nucleotides, guanosine 3′:5′-cyclic monophosphate (cGMP) and inosine 3′:5′-cyclic monophosphate (cIMP), had relatively little effect on [^{3}H]thymidine incorporation. Both cAMP and But$_2$cAMP caused a characteristic morphological change similar to that with ACTH, but no change in morphology was observed either with cGMP or cIMP. Adenosine nucleotides, 5′-AMP, ADP, and ATP, significantly inhibited [^{3}H]thymidine incorporation and caused a similar morphological change to that induced by cAMP or ACTH. Although other ribonucleoside monophosphates, CMP, GMP, and UMP, inhibited [^{3}H]thymidine incorporation, the extent of the inhibition was much less than that with adenine nucleotides and these nucleotides did not cause the morphological change in the cells. Since it has been reported that adenine nucleotides stimulate steroidogenesis in functional adrenal tumor cells (15), the content of cAMP in these cells was assayed after incubation with 1 mM 5′-AMP. Although ACTH increased the content of cAMP in the cells, 5′-AMP did not increase the cAMP concentration at all.

TABLE 3. *Effect of corticosteroids on [^{3}H]thymidine incorporation into DNA of functional adrenal tumor cells*

Corticosteroid	[^{3}H]Thymidine incorporation (cpm/bottle $\times 10^{-3}$)	(% inhibition)
Expt. 1		
None	162 ± 20	
Hydrocortisone	122 ± 16	25
Expt. 2		
None	216 ± 42	
Cortisone	193 ± 24	11
Prednisone	218 ± 45	0

Cells were incubated with corticosteroids (10 μg/ml of medium) for 6 hr. 2 Hr before the end of incubation, 10 μCi of [^{3}H]thymidine was added and [^{3}H]thymidine incorporation was assayed. Each group consisted of four–five cultures.

Effect of various doses of ACTH on steroidogenesis, [^{3}H]thymidine incorporation, and cAMP content

Because ACTH inhibited [^{3}H]thymidine incorporation and stimulated steroidogenesis, and cAMP inhibited [^{3}H]thymidine incorporation by functional adrenal tumor cells, it was of interest to study the relationship between these events. Steroidogenic response, [^{3}H]thymidine incorporation, and the cAMP content in these cells were compared after treatment with various doses of ACTH. For the determination of [^{3}H]thymidine incorporation, cells were incubated with ACTH for 6 hr, which was the optimum period for this determination (Fig. 1). However, the amount of corticosteroids in the medium was very low 6 hr after ACTH treatment, and steroidogenesis continued at a relatively constant rate up to 24 hr (Fig. 1). Therefore, cells were incubated with ACTH for 24 hr for the steroid assay. Because the content of

TABLE 4. *Effect of aminoglutethimide on steroidogenesis and [^{3}H]thymidine incorporation into DNA of functional adrenal tumor cells*

Treatment	Steroidogenesis (μg/ml)	[^{3}H]Thymidine incorporation (cpm/bottle $\times 10^{-3}$)
None	0.2 ± 0.1	302 ± 62
ACTH	2.9 ± 0.6	111 ± 11
ACTH + aminoglutethimide	0.4 ± 0.1	115 ± 18
Aminoglutethimide	0.0	262 ±26

One group of cells was incubated for 24 hr with ACTH (0.3 unit/ml) or aminoglutethimide (3.3 μg/ml) or both, and then the amount of corticosteroids in the medium was assayed. Another group of cells was incubated under the same condition for 6 hr. 2 Hr before the end of incubation, 10 μCi of [^{3}H]thymidine was added to each bottle and the incorporation was measured. Each group consisted of five cultures.

cAMP in these cells reached maximum values 30–60 min after ACTH treatment and gradually dropped thereafter, the content of cAMP was measured after incubation of the cells with ACTH for 30 min. As depicted in Fig. 2, the extent of stimulation of steroidogenesis and the amount of inhibition of [^{3}H]thymidine incorporation are closely related, and in parallel with the content of cAMP in these cells.

DISCUSSION

The present studies demonstrate that ACTH inhibits replication of functional adrenal tumor cells with a concomitant stimulation of steroidogenesis. One of the characteristic features of this phenomenon is that DNA synthesis in these cells is rapidly inhibited by ACTH. [^{3}H]Thymidine incorporation into DNA was decreased by about 50% 6 hr after ACTH treatment. Another characteristic feature is that DNA synthesis is rather specifically inhibited, whereas RNA and protein synthesis were not markedly changed. ACTH has no effect on [^{3}H]thymidine incorporation into DNA of human fibroblasts, which suggests that the inhibitory effect of ACTH on DNA synthesis is specific to cells derived from the adrenal cortex.

The possibility that the inhibition of DNA synthesis in these cells by ACTH is due to the accumulation of corticosteroids produced by the cells is unlikely for the following reasons. First, the time curve of [^{3}H]thymidine incorporation into DNA shows an early rapid decrease after ACTH treatment, when only a small amount of corticosteroids has accumulated in the medium. Second, a large excess of corticosteroids added to the culture medium does not significantly inhibit [^{3}H]thymidine incorporation. Third, ACTH inhibits [^{3}H]thymidine incorporation, even when steroidogenesis in these cells is blocked by aminoglutethimide.

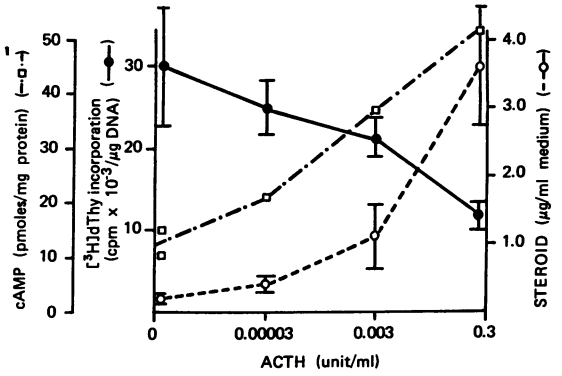

FIG. 2. Effect of various doses of ACTH on steroidogenesis, [^{3}H]thymidine incorporation, and the concentration of cAMP in functional adrenal tumor cells. For the steroid assay, cells were grown with the indicated concentrations of ACTH for 24 hr and the amount of corticosteroids in the medium was assayed. For the determination of [^{3}H]thymidine incorporation, cells were incubated with various concentrations of ACTH for 6 hr. 2 Hr before the end of incubation, 10 μCi of [^{3}H]thymidine was added and the incorporation was measured. Each point of the steroid assay and the determination of [^{3}H]thymidine incorporation represents the mean value of five cultures. For the cAMP assay, cells were incubated with ACTH for 30 min and the content of cAMP in cells was assayed. Each point represents one determination from five combined cultures.

TABLE 5. *Effect of nucleotides on [^{3}H]thymidine incorporation into DNA of functional adrenal tumor cells*

Nucleotide	[^{3}H]Thymidine incorporation (cpm/bottle × 10^{-3})	(% inhibition)	Morphological change
Expt. 1			
None	512 ± 78 (6)		−
ACTH	40 ± 14 (5)	92	+
But_2cAMP	85 ± 25 (6)	83	+
cAMP	204 ± 29 (6)	60	+
Expt. 2			
None	360 ± 83 (6)		−
cAMP	167 ± 31 (5)	54	+
cGMP	305 ± 75 (4)	15	−
cIMP	371 (2)	0	−
AMP	47 ± 12 (4)	87	+
ADP	102 ± 7 (4)	72	+
ATP	128 ± 16 (4)	64	+
Expt. 3			
None	398 ± 33 (6)		−
AMP	93 ± 12 (4)	77	+
CMP	226 ± 24 (4)	43	−
GMP	208 ± 45 (4)	48	−
UMP	282 ± 34 (5)	29	−

Cells were incubated with nucleotides (1 mM) for 6 hr. 2 Hr before the end of incubation, 10 μCi of [^{3}H]thymidine was added to each culture bottle and [^{3}H]thymidine incorporation was measured. Number of cultures for each group are shown in parentheses.

It is of special interest to know whether the inhibition of DNA synthesis by ACTH is mediated by cAMP. Both cAMP and But_2cAMP inhibit [^{3}H]thymidine incorporation and cause the same characteristic morphological change noted with ACTH. Both cGMP and cIMP have no effect on [^{3}H]thymidine incorporation and the morphology of these cells. The extent of stimulation of steroidogenesis and the amount of inhibition of DNA synthesis in response to various doses of ACTH are closely related, and are in parallel with the content of cAMP in these cells. These results strongly suggest that the inhibition of DNA synthesis by ACTH is mediated by cAMP.

It should be noted that adenine nucleotides, 5′-AMP, ADP, and ATP, inhibit [^{3}H]thymidine incorporation and cause a characteristic morphological change. Kowal and Fiedler have reported that these adenine nucleotides stimulate steroidogenesis in functional adrenal tumor cells (15). ACTH caused an increase in the cAMP content of these cells; however, we could not demonstrate an increase in the concentration of cAMP after treatment with 5′-AMP. It has been shown by Rall *et al.* that 5′-AMP causes an increase in the cAMP content of cerebral cortex slices of guinea pigs (23), but 5′-AMP does not increase the content of cAMP in bovine thyroid slices (24). How 5′-AMP might cause these phenomena without increasing the cAMP concentration is not known. One of the possibilities might be an effect on the energy metabolism of the cells caused by a change in the AMP concentration (25) or by a change in the balance among the adenine nucleotides (26). The relationship of the 5′-AMP effect to the cyclic nucleotide effect is unclear.

It is well known that differentiation and replication are antagonistic. Many differentiated cells in animal tissues do not

replicate, and tumor cells derived from differentiated tissues often lose their function. The growth of tumor cells of histologically differentiated types is generally slower than that of anaplastic types. In neuroblastoma, the differentiation of neurons is inversely related to the rate of cell division (27). In the absence of ACTH, functional tumor cells grow in tissue culture. Although these cells retain the differentiated function (i.e., steroidogenesis), they produce very little corticosteroid in the absence of ACTH. When ACTH is added to the medium, steroidogenesis in these cells is markedly stimulated, and concomitantly, the replication of these cells is almost completely inhibited. Both stimulation of steroidogenesis and inhibition of replication seem to be mediated by cAMP. This finding suggests that cAMP tends to cause these cells to regain the characteristics of normal adrenal cells. This is consistent with observations in transformed fibroblasts (8, 9) and Chinese hamster ovary cells (10, 11).

We reported that ACTH administration to animals stimulates DNA synthesis in adrenal glands (28). This effect of ACTH is probably mediated by cAMP (3). cAMP stimulates DNA synthesis and replication of rat thymus lymphocytes in culture (29). It is of interest to note that in certain normal cells replication seems to be stimulated by cAMP, whereas marked inhibition of replication by cAMP has been shown only with tumor cells. Thus, it appears that cAMP may play an important role in the control of replication, and may possibly be involved in carcinogenesis.

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