METABOLIC REGULATION OF STEROIDOGENESIS IN ISOLATED ADRENAL AND ADRENOCORTICAL CARCINOMA CELLS OF RAT. EFFECT OF ADRENOCORTICOTROPIC HORMONE AND ADENOSINE CYCLIC 3':5'-MONOPHOSPHATE ON THE PLASMA MEMBRANE

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1. Introduction

Normal isolated adrenal cell [1–3] and isolated adrenocortical carcinoma [4] cell preparations, having negligible phosphodiesterase activities [5,6], are useful in the direct study of comparative hormonal control mechanisms. Using the above two isolated cell systems as models, it has been demonstrated [4, 6–9]: a) that the biochemical lesion(s) responsible for the lack of stimulation of steroidogenesis by ACTH* or cAMP in the tumor cell is in the conversion of cholesterol to (20 S)-20-hydroxycholesterol; b) that the pregnenolone and progesterone incorporation into deoxycorticosterone and corticosterone is inhibited by ACTH in the tumor cells in contrast to the normal adrenal cells; c) that some, but not all of these actions of the hormone are found to be duplicated by cAMP. In the present investigation the effect of ACTH and its presumed mediator cAMP on deoxycorticosterone incorporation into corticosterone is compared in normal adrenal and adrenocortical carcinoma cells.

Although the stimulation of 11β-hydroxylase activity by cAMP in the adrenal homogenates and in the mitochondrial preparations of the rat have been reported by another laboratory [10,11], such stimulation in intact cells has not been found in this study.

* Trivial names and abbreviations: ACTH, adrenocorticotropic hormone; cAMP, adenosine cyclic 3':5'-monophosphate; Bt_2cAMP, N^6-2'-*O*-dibutyryl adenosine 3':5'-monophosphate; pregnenolone, 5-pregnen-3β-o1-20-one; corticosterone, 11β, 21-dihydroxy-4-pregnen-3, 20-dione; deoxycorticosterone, 21-hydroxy-4-pregnen-3, 20-dione.

2. Materials and methods

Isolated adrenal and adrenocortical carcinoma cell preparations were made as previously described [1–4]. The method of incubation with double labeled radioactive precursors was as previously described [7]: incubation was carried out in 3 Teflon flasks. Each flask contained 20 ml isolated adrenal tumor cell suspension [4] prepared from 1.5 g adrenal tumor tissue or the same amount of cell suspension obtained from 32 adrenals of the rat [1,2]. In addition, to the appropriate cell suspension, the first flask contained a mixture of 0.20 μCi of [4-^{14}C] deoxycorticosterone (specific activity 20–30 μCi/mmole) and 4.0 μCi corresponding [1,2-$^3H_2$] deoxycorticosterone (specific activity 30–50 Ci/mmole) (^{3}H/^{14}C ratio, 20.00); the second flask contained 0.20 μCi of [^{14}C] deoxycorticosterone and the third flask 4.0 μCi of [^{3}H] deoxycorticosterone + ACTH (200 μU/ml) or Bt_2cAMP (1 mM). The incubation was continued for 150 min and the reaction was stopped by the addition of 15 ml of distilled water and 75 ml of methylene chloride into each flask. The contents of the second flask which contained [^{14}C] deoxycorticosterone were mixed with the contents of the third flask which contained [^{3}H] deoxycorticosterone + ACTH or Bt_2cAMP. To the reaction mixture of the two flasks 15 mg of corticosterone was added and the products processed

identically.

The experiments with the preloading of the cells with [^{3}H] deoxycorticosterone were conducted as previously described [12]; 20 ml of suspended cells obtained from adrenals of 32 rats were preincubated for 30 min with 8.0 μCi of [^{3}H] deoxycorticosterone + 5 mg of non-radioactive deoxycorticosterone; the cell pellet obtained by centrifugation [1,2] was washed with 20 ml Krebs–Ringer–bicarbonate buffer which contained albumin and glucose; the cells were resuspended in 40 ml of fresh Krebs–Ringer–bicarbonate buffer containing albumin and glucose. One incubation flask contained 20 ml of cell suspension with ACTH or Bt_2cAMP. The incubation was carried out for 150 min and the reaction mixtures processed as mentioned previously except to the reaction mixture of each flask both deoxycorticosterone (15 mg) and corticosterone (15 mg) were added.

Deoxycorticosterone and corticosterone were purified by thin-layer chromatography [7,8,12]. The isolated deoxycorticosterone was acetylated [7,13], further purified by thin-layer chromatography [7, 8, 12] and crystallized from acetone-*n*-hexane to constant specific activity. The purified corticosterone was crystallized from acetone–ligroin until the specific activity and (^{3}H/^{14}C) ratio were constant.

Table 1

Effect of ACTH on the incorporation of [^{3}H] deoxycorticosterone into corticosterone in normal isolated adrenal and adrenocortical carcinoma cells.

Isolated cells from	Crystallization	[^{3}H/^{14}C] ratio of corticosterone from	
		Flask 1	Flask 2 + Flask 3
Normal adrenal	1st	20.00	24.12
	2nd	20.06	25.70
	3rd	20.59	26.49
Adrenocortical carcinoma	1st	19.97	27.96
	2nd	17.76	26.71
	3rd	16.40	25.20

The [^{3}H/^{14}C] ratios of corticosterone obtained after the incubation of [4-^{14}C, 1,2-$^3H_2$] deoxycorticosterone with isolated adrenal and adrenocortical carcinoma cells. Incubation was carried out in 3 flasks containing 20 ml isolated adrenal or adrenocortical carcinoma cell preparation as mentioned in Materials and methods. Flask 1 contained a mixture of [1,2-$^3H_2$] deoxycorticosterone (4.0 μCi) + [4-^{14}C] deoxycorticosterone (0.20 μCi) (^{3}H/^{14}C ratio 20.00); flask 2 contained [1,2-$^3H_2$] deoxycorticosterone (4.0 μCi) + ACTH (200 μU/ml) and flask 3 contained [4-^{14}C] deoxycorticosterone (0.20 μCi). The incubation was 2.5 hr and the reaction was stopped by the addition of 75 ml methylene chloride to each flask. The contents of the second and third flask were mixed and corticosterone isolated as described in Materials and methods.

3. Results

3.1. *Effect of ACTH on the incorporation of deoxycorticosterone into corticosterone in isolated adrenal and adrenocortical carcinoma cells*

Studies carried out in the normal adrenal and adrenocortical carcinoma cells where one group of cells was incubated with [1,2-$^3H_2$] deoxycorticosterone + ACTH and the other group with [4-^{14}C] deoxycorticosterone alone followed by mixing the two incubation solutions after terminating the incubation, showed (table 1), 26% stimulation by the hormone in the isolated adrenal cell and 47% stimulation in the adrenocortical carcinoma cells.

In order to determine if the above effect of ACTH represents stimulation of the enzyme or of the transport mechanisms in the plasma membrane, the isolated adrenal and adrenocortical carcinoma cells were preincubated with [1,2-$^3H_2$] deoxycorticosterone and the effect of ACTH observed on these cells. Table 2, shows that the preloading of the cells eliminated the stimulation by ACTH in both normal and tumor cells. This shows that the stimulatory effect of the hormone was at the transport level of the plasma membrane of the normal adrenal and the adrenal tumor cell.

To eliminate the possibility that the non-stimulatory effect of ACTH in the preloaded cells might be due to the insufficient availability of the substrate, deoxycorticosterone, the latter steroid was isolated, purified and crystallized as the 21-acetyl derivative. The results in table 2 show that there was still more than 80% of the unused substrate, deoxycorticosterone, in the incubation system.

Table 2
Effect of ACTH on the incorporation of [^{3}H] deoxycorticosterone into corticosterone in preloaded normal isolated adrenal and adrenocortical carcinoma cells

Isolated cells from	Compound	Crystallization	[^{3}H] dpm of compound from	
			Control	+ ACTH
Normal adrenal	Deoxycorticosterone acetate	Crude	1 239 000	1 245 000
		1st	1 230 000	1 240 000
	Corticosterone	1st	206 500	243 000
		2nd	202 000	222 000
		3rd	200 400	219 000
Adrenocortical carcinoma	Deoxycorticosterone	1st	1 962 000	1 905 000
	Corticosterone	1st	69 500	66 100
		2nd	68 400	60 300

The total [^{3}H] dpm of the products (and their derivatives) obtained after the incubation of [1,2-$^3H_2$] deoxycorticosterone with isolated adrenal and adrenocortical carcinoma cells. 20 ml of isolated adrenal cell preparation obtained from adrenals of 32 rats or the same amount of cell suspension obtained from 1.5 g adrenal tumor was preincubated with 5 mg of non-radioactive deoxycorticosterone + [1,2-$^3H_2$] deoxycorticosterone (8.0 μCi) for 30 min. The cells were washed and resuspended in 40 ml of Krebs–Ringer–bicarbonate buffer containing albumin and glucose.

20 ml aliquot of cell suspension was added into one flask and the second flask contained 20 ml aliquot of cell suspension + ACTH (200 μunits/ml). The incubation was for 2.5 hr and the reaction was stopped by the addition of 75 ml methylene chloride to each flask. Deoxycorticosterone and corticosterone were isolated as described in Materials and methods.

3.2. *Effect of Bt_2cAMP on the incorporation of deoxycorticosterone into corticosterone in isolated adrenal and adrenocortical carcinoma cells*

Previous studies [1,7] have shown that Bt_2cAMP is a much better stimulator of steroidogenesis than cAMP in the isolated adrenal cell. It was demonstrated, there, that the mode of action of both of these cyclic nucleotides is the same. In order that ACTH in its effect upon the incorporation of deoxycorticosterone into corticosterone might be compared with its presumed mediator, cAMP, studies were conducted in the presence of Bt_2cAMP on the conversion of [1,2-$^3H_2$] deoxycorticosterone to corticosterone. A shown in table 3, this nucleotide, like ACTH, stimulated (34%) the incorporation of deoxycorticosterone into corticosterone into normal adrenal cells. Further, analogous to the ACTH effect, the preloading of isolated adrenal cells with [1,2-^{3}H] deoxycorticosterone eliminated the stimulatory effect of deoxycorticosterone (table 4). This shows that the effect of Bt_2cAMP was also at the transport level of the plasma membrane. A total of 80 to 85% unused deoxycorticosterone was isolated from the incubation mixture, thus showing that marked diminution of the total substrate cannot explain the observed results.

Table 3
Effect of Bt_2cAMP on the incorporation of [^{3}H] deoxycorticosterone into corticosterone in normal isolated adrenal and adrenocortical carcinoma cells

Isolated cells from	Crystallization	[^{3}H/^{14}C] ratio of corticosterone in	
		Flask 1	Flask 2 + Flask 3
Normal adrenal	1st	20.04	25.47
	2nd	20.06	27.11
	3rd	20.35	28.49
Adrenocortical carcinoma	1st	20.00	21.47
	2nd	19.91	22.72
	3rd	19.86	22.57

The [^{3}H/^{14}C] ratios of the products obtained after the incubation of [4-^{14}C,1,2-$^3H_2$] deoxycorticosterone with isloated adrenal and adrenocortical carcinoma cells. Conditions of the experiment were similar to the experiment in table 1.

The results in table 3 show that, in constrast to the stimulatory effect of ACTH and Bt_2cAMP in the normal adrenal cell and also in contrast to the stimulatory effect of ACTH in the adrenal tumor cell, Bt_2 cAMP did not have any stimulatory effect upon the deoxycorticosterone incorporation into corticosterone by the tumor cells.

Although the results reported are from single experiments, they have been confirmed in others. The absolute values have varied from experiment to experiment but the relationship of the individual results were consistent between experiments.

Table 4
Effect of Bt_2cAMP on the incorporation of [^{3}H]deoxycorticosterone into corticosterone in preloaded normal isolated adrenal cells.

Compound	Crystallization	[^{3}H] dpm of compound from	
		Control	+ Bt_2cAMP
Deoxycorticosterone acetate	Crude	1 177 000	1 182 000
	1st	1 169 000	1 178 000
Corticosterone	1st	187 800	178 800
	2nd	183 500	177 600
	3rd	182 300	184 000

The total [^{3}H]dpm of the products (and their derivatives) obtained after the incubation of [1,2-$^3H_2$]deoxycorticosterone with isolated adrenal cells. Conditions of the experiment were similar to the experiment in table 2.

4. Discussion

There is strong evidence that the stimulation of steroidogenesis by ACTH in the normal adrenal cell is mediated by cAMP [14–17]. That the stimulatory activity of this nucleotide is on the rate limiting step, cholesterol to pregnenolone, has been demonstrated [15,18,19] and supported by this laboratory [1,2,7]. The hypothesis [12] of Garren et al. [16,20], concerning the mode of action of ACTH in the adrenal cell has been modified [12] in the light of new evidence, some of which is presented herein, that modification involves the inclusion especially in the adrenal tumor, of aspects of hormonal action which are independent of mediation by cAMP.

The present studies conducted with the isolated adrenal and adrenocortical carcinoma cell demonstrate a unique effect of ACTH, which consists in the stimulation of deoxycorticosterone incorporation into corticosterone. The evidence has been provided that this requires the interposition of the plasma membrane since preloading the cells eliminates hormonal stimulation. It is, therefore, most probable that this effect is dependent on the transport of deoxycorticosterone across the plasma membrane of the normal and malignant adrenal cell. In the normal adrenal cell, Bt_2cAMP duplicates the stimulatory effect of ACTH; and like the latter agent, the stimulation by Bt_2cAMP of deoxycorticosterone incorporation into corticosterone is shown to be at the transport level of the plasma membrane.

Of particular interest, however, are the results obtained with the adrenocortical carcinoma cell, where, although, ACTH stimulates the transport of deoxycorticosterone into the cell, Bt_2cAMP lacks any stimulatory activity. This suggests that the change of the normal adrenal cell to the adrenal tumor cell leads to considerable modification of biochemical control processes at the membrane level which in turn leads to modification of intracellular steroid concentrations and their resultant metabolism. Indirect evidence that there are differences in the rates of plasma or mitochondrial metabolism of (20 S)-20-hydroxycholesterol and pregnenolone between the adrenal tumor and normal adrenal has been provided earlier [8]. The present studies showing that ACTH stimulates the transport of deoxycorticosterone into the cell may be quite important in view of the recent finding that the biosynthetic step, deoxycorticosterone to corticosterone, is a relatively slow step in the normal adrenal cell [12]. The implication is that if the hormone can stimulate the transport of deoxycorticosterone across the membrane it can also transport the precursor out of the cell. The hormone could, therefore, regulate the availability of the substrates for various enzymic reactions. Since this action of the hormone can be mimicked by Bt_2cAMP, it may be inference be suggested that the action of the hormone is being mediated by cAMP. Since the stimulatory effect of ACTH is not duplicated by Bt_2cAMP in the tumor, it is suggested that the membrane phenomena in the malignant cell may be dependent on other factors, and possibly on other cyclic nucleotides. Evidence has been provided earlier that in the same tumor, ACTH inhibits pregnenolone incorporation into corticosterone and this is distinctly different from the effect of cAMP [7].

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