PREPARATION OF ADRENOCORTICAL CELL SUSPENSION HIGHLY RESPONSIVE TO ACTH OR DIBUTYRYL-CYCLIC AMP

Effects of albumin, Ca^{2+} or stress

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1. Introduction

Although the cells of the adrenal cortex can synthesize cholesterol from acetate [1] most of the cholesterol used for synthesis of corticosteroid hormones derives from plasma [2–4]. Free and esterified cholesterol enter the adrenal cell in a form of a lipoprotein [5] either of high [6–8] or low density [9,10]. Chronic administration of corticotropin (ACTH) has been shown to stimulate not only the entry of plasma cholesterol into the adrenal cells, but also its utilization for steroid hormone synthesis [5,6,8,9], as well as the hydrolysis of cholesterol esters [11,12]. Initial studies on lipoprotein cholesterol uptake by the adrenal cortex were made in quartered glands [6] and in isolated cells in culture, either from normal adrenal [8,9], or adrenal tumor [8–10]. A better approximation to in vivo conditions seems to be the investigation of cholesterol uptake, and its utilization for steroid hormone synthesis, by freshly isolated cells. These however require a preparation containing only cells of zona fasciculata-reticularis, undamaged and highly responsive to ACTH or dibutyryl-cyclic AMP, devoid of erythrocytes and/or other types of cells. Here we have attempted to prepare such a suspension.

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2. Materials and methods

2.1. *Reagents*

Corticosterone, dexamethasone, N^6-2-*O*-dibutyryl cyclic 3′,5′-AMP, bovine serum albumin fraction V (BSA), and collagenase (batch 2139) were products of Sigma (USA). Synthetic $ACTH_{1-24}$ (Synactene) was purchased from Ciba-Geigy (Switzerland). Other reagents were products of Prolabo (France).

2.2. *Animal treatment*

Female rats of Wistar strain (~200 g body wt), kept in single cages, were fed a standard diet (Extralabo, Provins). Control and stressed animals were killed by decapitation. Experimental animals received dexamethasone (10 μg/ml drinking water) 12 h prior to killing. All experiments were started at 9 a.m. The experimental animals were anesthesized by i.p. injection of sodium pentobarbital. The blood was withdrawn and the viscera were perfused through the abdominal aorta with five 12 ml portions of oxygenated Krebs-Ringer bicarbonate buffer (KRB) (pH 7.4) containing 0.2% glucose. Adrenal 'cores' consisting of zona fasciculata-reticularis and medulla were obtained, whereas 'capsules' were discarded [13].

2.3. *Preparation of the adrenal cell suspension*

Glassware used for the preparation of cell suspension and incubations was washed with chromic acid and siliconized with Silicone DC-87 Drifilm (Pierce, Rocheford). Adrenal cell suspensions were prepared as in [14] with some minor changes. The cores were quartered with a razor blade and placed in a conical flask containing 0.24% collagenase in 0.8 ml/2 adrenals

of KRB (pH 7.4) containing 0.2% glucose and 1% albumin (KRBGA). The flasks were stoppered, gassed for 10 s with 95% O_2:5% CO_2, and incubated for 60 min at 37°C in a shaking water bath. After 20 and 40 min the dispersion medium was collected, fresh medium added, and the tissue was passed through a 5 ml plastic syringe without a needle. Oxygenating was repeated every 20 min. The tissue was finally dispersed by flushing 3 times through a 5 ml plastic syringe with a 21 gauge needle. The suspension was filtered through a nylon gauze 100 μm mesh, and centrifuged at room temperature for 2 min at 120 × *g*. The pellet was washed 3 times with 6 ml portions of KRBGA, and finally filtered through a nylon gauze. A sample of the filtrate was taken for counting the cells, and their viability was estimated by a trypan blue exclusion technique.

2.4. *Incubation of aliquots of cell suspension with test substances*

Test substances were added in KRBGA followed by the addition of cell suspension (100–200 × 10^3 cells/ml medium). The samples were oxygenated for 10 s and incubated at 37°C in a shaking water bath. Every 15 min of incubation the oxygenation was repeated. The incubations were stopped by the addition of dichlormethane and corticosterone concentration was estimated in samples of cells plus medium [15].

2.5. *Other methods*

Lipids were extracted from the adrenal cells [16] and free and esterified cholesterol separated and quantitated [17]. Adrenocortical cells were centrifuged and washed twice with 10 ml portions of KRBG to remove BSA, and protein concentration was determined [18].

3. Results

3.1. *Evaluation of the quality of cell preparation*

A number of isolated cells amounted to 0.6–0.8 × 10^6/2 adrenal glands, 95% undamaged, and 92.3 ± 0.8% viable (mean ± SEM, 27 expt). About 80% of the cells had the feature of zona fasciculata cells and the remaining 20% were zona reticularis cells. Red blood cells in the preparation amounted to 34 ± 6/1000 adrenal cells. The cholesterol of the red blood cells represented 0.02% of the free cholesterol of the adrenocortical cells. No other types of cells were noticed.

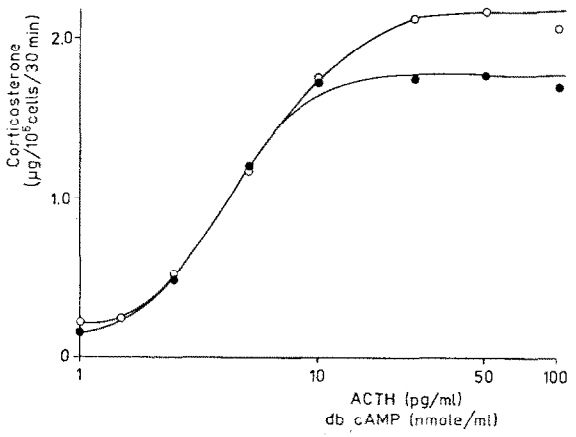

Fig.1. Stimulation of corticosterone output by ACTH or db cAMP in isolated adrenocortical cells. Adrenocortical cells, prepared from rats killed by decapitation were incubated for 30 min at 37°C in KRBGA buffer (pH 7.4) containing 7.65 mM $CaCl_2$. (•) ACTH; (○) db cAMP.

Oxygen uptake, measured by an oxygen electrode [19] did not change during 4 h incubation of the cells at 37°C in KRBGA, and was not affected by addition of 1 mM succinate or 1 mM $NADPH_2$. Both the number of cells, and their viability did not change during the incubation period.

Cholesterol ester concentration within the cells amounted to 294.8 ± 51.7 and free cholesterol to 37.0 ± 4.7 nmol/10^6 cells, i.e., 790.4 ± 95.3 and 111.0 ± 8.9 nmol/mg protein, respectively.

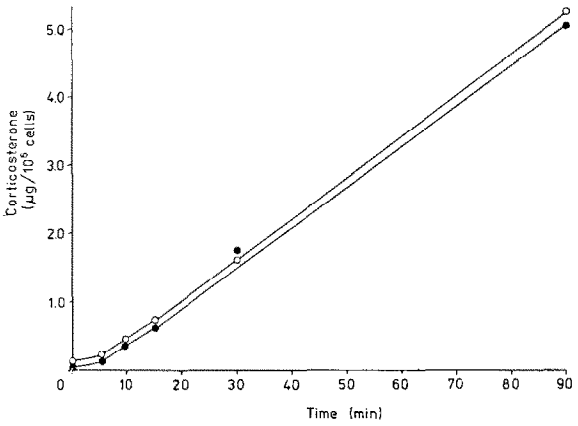

Fig.2. Time course of corticosterone output in ACTH or db cAMP stimulated adrenocortical cells. Conditions as described in footnote to fig.1 except for various time of incubation. (•) ACTH (500 pg/ml); (○) db cAMP (1000 nmol/ml).

3.2. *Dose–response and time course of corticosterone output in ACTH or db cAMP stimulated cells*

Corticosterone output, measured as a function of either ACTH or db cAMP concentration showed a sigmoid dose–response with almost the same maximal corticosterone output (fig.1).

Corticosterone output measured as a function of time at saturating concentration of either ACTH on db cAMP showed a straight line kinetics up to 90 min incubation. However a deviation from the straight line was noticed at short incubation periods up to 10 min for both ACTH or db cAMP (fig.2).

3.3. *Effect of albumin*

Albumin concentration in the incubation medium did not affect to a large extent either basal (B_0) or maximal (B_{max}) corticosterone output. Typical dose–response curves are shown in fig.3a. Concentration of ACTH giving half of the maximal corticosterone output A_{50} amounted to 4.5 and 4.6 pg/ml, respectively, for samples incubated in the presence of 0.5 and 1% albumin.

3.4. *Effect of calcium*

Stepwise increases of calcium concentration in the incubation medium at 1% albumin did not change to a large extent either basal or maximal corticosterone output. However a marked decrease of B_{max} was noticed in samples incubated without calcium (fig.4a). The values of A_{50} rose to 4.5, 7.0, 8.5 and 17.0 pg/ml for [$CaCl_2$] of 7.65, 2.5, 1.25 and 0.0 mM, respectively.

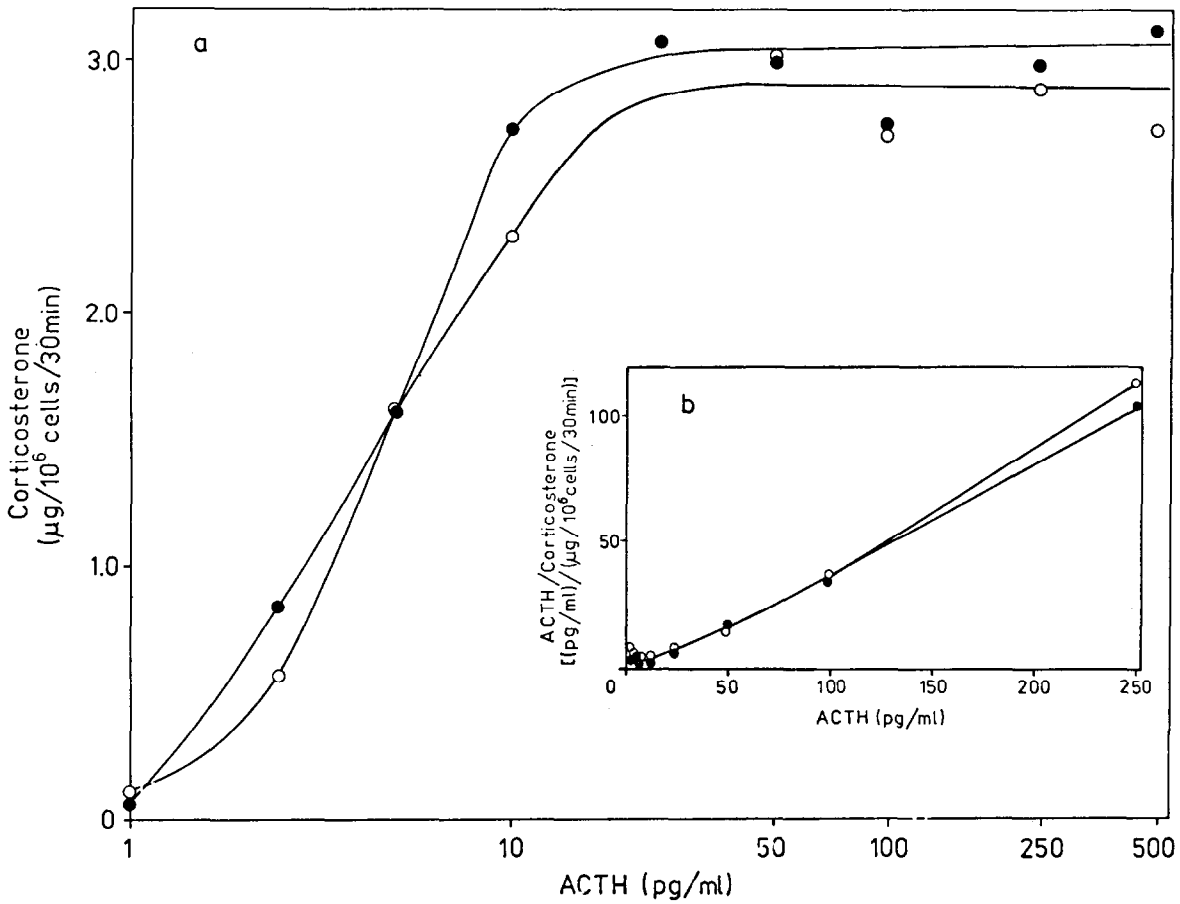

Fig.3. Effect of albumin on corticosterone output as a function of ACTH concentration. Adrenocortical cells, prepared from dexamethasone pre-treated rats were incubated as described in footnote to fig.1 except for albumin concentration: (○) 0.5% albumin; (●) 1% albumin. (a) Dose–response curves at different albumin concentration; (b) evaluation of the response at different albumin concentration.

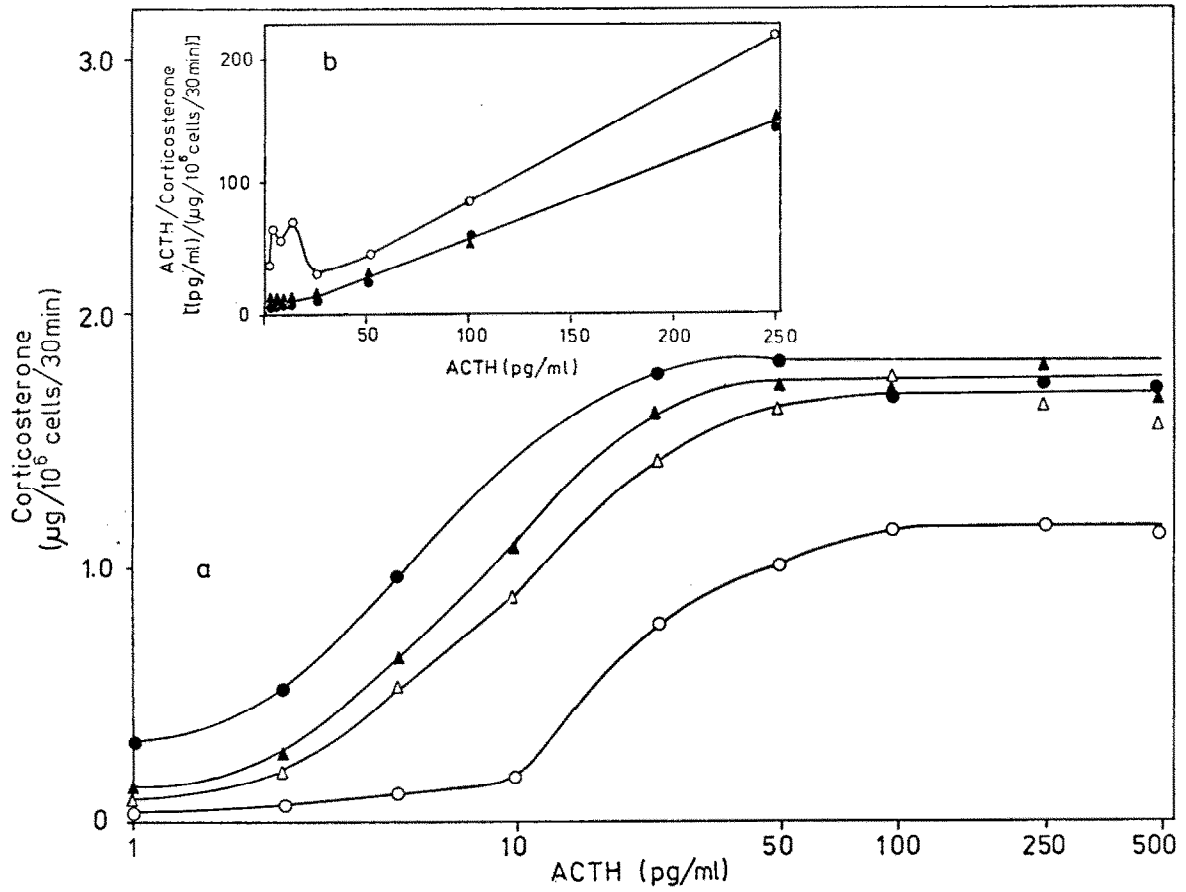

Fig.4. Effect of Ca^{2+} on corticosterone output as a function of ACTH concentration. Adrenocortical cells, prepared from dexamethasone pre-treated rats were incubated as described in footnote to fig.1 except for various $CaCl_2$ concentrations: (○) no calcium; (△) 1.25 mM calcium; (▲) 2.5 mM calcium; (●) 7.65 mM calcium. (a) Dose–response curves at different calcium concentration; (b) evaluation of the response at different calcium concentrations.

Table 1
Effects of calcium depletion or stress on corticosterone output in isolated adrenocortical cells

Parameter tested	Unstressed		Stressed		Dexamethasone pre-treated			
					2.5 mM $CaCl_2$ washed		$CaCl_2$ free washed	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Basal output (B_0)	0.03	0.04	0.60	0.61	0.03	0.04	0.03	0.03
Maximal output (B_{max})	2.92	3.20	1.76	1.74	2.70	2.84	1.76	1.74
A_{50}	4.40	4.20	2.50	–	4.10	4.60	4.50	4.40
Fold stimulation	97.3	80.0	2.9	2.7	90.0	70.9	58.7	58.7

The animals were either kept intact, pretreated with dexamethasone and anaesthetized, or stressed by exposing to noise for 2 h prior to killing. Two cells preparations from dexamethasone pre-treated animals were obtained with or without the addition of $CaCl_2$ for the final wash of the cells. Cell preparations were incubated as in fig.1. A_{50} is the concentration of ACTH giving half of the maximal corticosterone output

3.5. Effect of calcium depletion or stress

Pre-treatment of the animals with dexamethasone did not affect either basal or maximal corticosterone output in isolated adreno-cortical cells, and the sensitivity to ACTH, measured by A_{50}, remained unchanged (table 1). In stressed animals 15–20-fold elevation of B_0 was observed, as compared with intact control animals, and B_{max} was lower by ~40% of the value obtained in the cell suspension prepared either from intact or dexamethasone pre-treated animals. In the cell suspensions prepared without the addition of $CaCl_2$ for the final wash, B_{max} was also reduced, but there was no change in A_{50} as compared with the group of intact animals.

4. Discussion

Preparation of the adrenal cells seemed to fulfil the criteria required for studying the effect of ACTH on steroidogenesis. The preparation contained only fasciculata-reticularis cells, undamaged judging from their morphological appearance and the lack of stimulation of oxygen uptake by addition of either succinate or $NADPH_2$ into the incubation medium. Viability of the cells, measured by oxygen consumption or exclusion of trypan blue, did not change over 4 h incubation, which argues for a 'good quality' of cell preparation. The isolated cells contained about 37 and 300 nmol/10^6 cells of free and esterified cholesterol, respectively, which was consistent with [8,20], but lower than the value in [21]. Small increment of the erythrocyte cholesterol should not affect the results of the studies on cholesterol uptake by isolated adrenal cells.

The linearity of dose–response curves between 1 and 10 pg ACTH/ml with A_{50} ~5 pg/ml offered the possibility to develop a sensitive bioassay for human serum ACTH. The assay in [22] was not widely used because of either low responsiveness of the cells (high A_{50}), which would require large volumes of serum, or preparing adrenocortical cell suspension from hypothysectomized rats to lower A_{50} [22].

High rate of corticosterone output observed under ACTH or db cAMP stimulation (~6 μg . 10^6 cells^{-1} . 60 min^{-1}) compared favourably with most of the reports which gave lower values of B_{max} [13,21,22]. However it was possible to increase the reaction rate by ~30% by preincubating the cells prior to stimulation by ACTH [23], and this offers further possibility to increase responsiveness of the cell preparation reported.

The addition of albumin was shown to improve the responsiveness of adrenal cell preparations to ACTH [13,14,22,24]. However we had not observed any effect of lowering albumin concentration from 1.0–0.5% either on B_{max} or A_{50} (fig.3a) except of the deviation from the straight line (fig.3b) which is a feature of 'bad quality' of cell preparation [13]. On the contrary, diminution of calcium concentration resulted in the decrease of B_{max} and increase in A_{50} values in ACTH stimulated cells (fig.4a) the most dramatic changes being observed in the suspension of the cells incubated without calcium. Results plotted as suggested [13] gave straight lines only when the cells were incubated in a medium containing 7.65 mM $CaCl_2$ (fig.4b). Application of lower calcium concentration caused marked deviation from the straight line which suggest that the responsiveness of the cells is greatly decreased. However no effect of lowering calcium concentration was noticed when the cells were stimulated with db cAMP, which was consistent with [25,26].

When the cells were prepared from the adrenal glands of stressed rats, an increase in basal corticosterone output was observed (table 1). Despite low value of A_{50} suggesting high responsiveness of the cells to ACTH maximal corticosterone output was lower by ~40% as compared with intact animals and this figure compares favourably with the B_{max} found in the cells devoid of calcium by washing with calcium-free medium. Lowering of B_{max} might reflect depletion of cholesterol stores within the adreno-cortical cells which followed ACTH stimulation [10,21]. However, direct measurements of cholesterol and cholesterol ester concentration within the unstimulated and stimulated adrenal cells (not shown) did not support this supposition. There is a possibility that the decrease in B_{max} observed in preparations from stressed animals might be related to the depletion of the cells in Ca^{2+}, but this should be confirmed by direct measurements of calcium concentration within the cells.

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