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Stereological and functional investigations on isolated adrenocortical cells. III. Zona glomerulosa cells of chronically ACTH-treated rats*

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INTRODUCTION

In a previous investigation we described the morphology and secretory activity of isolated adrenocortical cells obtained from normal rats (Andreis *et al.* 1989). Outer subcapsular (zona glomerulosa [ZG]) cells were found to display morphological features resembling those of parenchymal elements *in situ* and to possess a basal secretion of aldosterone, corticosterone and progesterone. They also responded to acute exposure to ACTH, the main stimulator of steroid synthesis in isolated adrenocortical cell preparations (Vinson, Hinson & Raven, 1985), by increasing aldosterone and corticosterone release (and decreasing that of progesterone).

It is current knowledge that prolonged exposure to high levels of circulating ACTH causes a notable hypertrophy of rat ZG cells *in vivo* (Nussdorfer, Mazzocchi & Rebuffat, 1973; Nussdorfer *et al.* 1977; Nussdorfer, 1986), which, however, seems to be coupled with a drop in the plasma concentration of aldosterone (Müller, 1978; Aguilera, Fujita & Catt, 1981; Vazir, Whitehouse, Vinson & McCredie, 1981; Riondel *et al.* 1987). As far as we know, no investigations are available which aim at correlating the structure and function of isolated ZG cells of chronically ACTH- pretreated rats.

MATERIALS AND METHODS

Animal treatment and preparation of dispersed cells

Adult male rats of Wistar strain (250 ± 20 g body weight) were used. They were maintained on a standard diet (Rat-Mouse Chow; Zoofarm, Padua, Italy) with tap water *ad libitum* until they were killed. The animals were divided into two groups, one of which was given daily subcutaneous injections of 10 IU kg^{-1} ACTH (Synacthen depot; Ciba, Milan, Italy) for 5 days. The other group received daily subcutaneous injections of the oil vehicle and served as controls. The rats were killed by decapitation between 9.00 and 10.00 am, and their trunk blood was collected. Aldosterone and corticosterone were extracted from plasma and purified (Sippell *et al.* 1978) and their concentrations were measured by radioimmunoassay (Aldo RIA Kit, Diagnostic Products, Los Angeles, USA; Corticosterone RIA Kit, Eurogenetix, Milan, Italy).

Adrenal glands were promptly removed and freed of adherent fat. The capsule was gently stripped off in order to isolate the ZG (Tait, Tait, Gould & Mee, 1974).

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Dispersed ZG cells were prepared by collagenase/DNase digestion, according to Szalay (1981). Twenty adrenal pairs were used for each group. The details of the isolation procedure have been given previously (Andreis *et al.* 1989). Final cell pellets were immediately employed for functional and morphological studies. Inner cell contamination in capsular cell preparations was checked by electron microscopy (inner adrenocortical cells always contain mitochondria with vesicular cristae and numerous lipid droplets; see below for ZG cells) and found to be less than 5–6%.

Functional studies

Samples of isolated cells from both groups of rats were suspended in a mixture of Medium 199 (DIFCO Labs, Detroit, USA) and potassium-free Krebs–Ringer bicarbonate buffer (2:1, v/v), containing 5 g l^{-1} human serum albumin. Aliquots of cell suspensions (2×10^5 cells ml^{-1}) were incubated in groups of 6 with ACTH 10^{-8} M (Sigma, St. Louis, USA) or without ACTH. The incubation was carried out in a shaking bath at 37 °C for 90 minutes, in an atmosphere of 95% O_2 and 5% CO_2. At the end of the experiment, the incubation tubes were centrifuged (200 ***g*** for 20 minutes) at 4 °C, and the steroid concentrations in the supernate were assayed by HPLC.

One hundred nanograms of dexamethasone were added to the samples (1–2 ml) to act as an internal standard, and the samples were then extracted with 10 ml dichloromethane. The extracts were washed twice with 2 ml 0·1 M-NaOH and 1 ml distilled water and then evaporated to dryness under vacuum and redissolved in 50 μl methanol. The recovery of steroids was 85 ± 8% (S.D.). The samples were injected via a Rheodyne 7105 valve in a Perkin–Elmer liquid chromatograph (Ser. 2/2) and steroids were assayed according to the technique of O'Hare, Nice, Magee-Brown & Bullman (1976), as described in a previous paper (Andreis *et al.* 1989). Steroids were detected at 240 nm wavelength, and the minimum detectable quantity was about 5 ng. All the reagents employed were of HPLC grade (Baker Chemical, Deventer, Holland) and were degassed under vacuum before use. Standard steroid hormones were purchased from Sigma.

Morphological studies

Samples of the dispersed cells were centrifuged at 200 ***g*** for 20 minutes, and the resultant loose pellets were fixed in 3% glutaraldehyde in 0·1 M cacodylate buffer (for 40 minutes), postfixed in 1% osmium tetroxide in 0·1 M phosphate buffer (for 60 minutes) and embedded in Epon, as described by Szabo, Szalay & Toth (1984).

Eight plastic blocks for each group were examined. From each block, two thick (0·5 μm) and adjacent thin (80 nm) sections were cut with an LKB III ultramicrotome. Isotropic sectioning was guaranteed by the fact that isolated sphere-like randomly-orientated cells were cut. For each series of thick sections, five light micrographs at a final magnification of × 1000 were taken. Thin sections were examined in a Hitachi H-300 electron microscope at a direct magnification of × 7000. Six electron micrographs at a final magnification of × 21 000 and four electron micrographs at a final magnification of × 42 000 were taken for each series of thin sections.

The average volume of the isolated cells was determined by calculating their mean diameter, using the Schwartz correction for spheres (Elias & Hennig, 1967). The bias introduced by the fact that isolated cells were not true spheres presumably affected control and ACTH-treated cells to the same degree, since the experimental treatment did not induce any significant change in axial ratios of cells (control cells, 1·302 ± 0·22 S.D.; ACTH-treated cells, 1·290 ± 0·20 S.D.; *P* not significant, n = 14). The volume densities (V_v) of nuclei, mitochondrial compartment, membrane space (i.e. the cellular

space occupied by endoplasmic reticulum membranes, including Golgi apparatus), lipid-droplet compartment and dense-body compartment were evaluated on the electron micrographs at ×21000, using a square lattice test system of Type A (100 test points/100 cm^2) (Weibel, 1979). The surface densities (S_v) of mitochondrial cristae and agranular endoplasmic reticulum (AER) tubules were determined according to Loud (1962), using a parallel-line test system (100 cm of test line/100 cm^2). S_v values were corrected for section-thickness effect, by assuming the following geometry: mitochondrial cristae in control cells, discs; mitochondrial cristae in ACTH-treated cells, tubules; AER in both cell groups, tubules (Weibel & Paumgartner, 1978). S_v values were not corrected for image loss due to membrane tilt with respect to the electron beam axis, but we assumed that the bias was identical in control and ACTH-treated cells. V_v and S_v were transformed in absolute values per cell by multiplying them by the average cell volume; the analysis of covariance between densities and cell volume justified this procedure. Each morphometric parameter was the mean of eight separate estimates.

Statistical treatment of results

The data were averaged for each experimental group and the standard deviation (S.D.) of the mean was calculated. The statistical comparison of the data was performed by ANOVA, Student's *t* test and the multiple range test of Duncan (Bliss, 1967).

RESULTS

Prolonged ACTH treatment caused a 52% rise in the basal blood concentration of corticosterone and a 32% decrease in that of aldosterone (Table 1).

Isolated ZG cells of control rats were round or slightly ovoid elements, about 10 μm in diameter, with round nuclei. They contained ovoid or irregularly shaped mitochondria with tubulo-laminar cristae (Fig. 1), some AER profiles and a few small lipid droplets. After ACTH pretreatment of rats, ZG cells always contained ovoid or elongated mitochondria with numerous tubulo-convolute cristae (Fig. 2). Other ultrastructural features did not display evident changes.

Stereology showed (Table 2) that ACTH pretreatment provoked a significant increase in the average volume of ZG cells (58%) and nuclei (46%). Cell hypertrophy was associated with significant rises in the volumes of the mitochondrial compartment (61%) and membrane space (88%), as well as in the surface area per cell of mitochondrial cristae (91%) and AER (97%). The ACTH-induced transformation of cristae from a tubulo-laminar configuration to a tubulo-convolute one was coupled with a rise in their S_v from 13·8 to 16·4 ($\mu m^2/\mu m^3$ of mitochondria). The volume of the lipid droplet compartment was decreased (−47%), while that of the dense body compartment did not undergo any significant change.

The secretory activity of isolated ZG cells is shown in Table 3. Zona glomerulosa cells of control rats possessed an appreciable basal secretion of progesterone, corticosterone, 18-hydroxycorticosterone and aldosterone. ACTH pretreatment of rats significantly decreased basal secretion of progesterone (−50%) and corticosterone (−52%), and completely annulled that of 18-hydroxycorticosterone. Conversely, it provoked a 90% rise in the basal production of aldosterone.

Acute ACTH exposure caused rather similar effects on the secretory activity of ZG cells of both control and ACTH-pretreated rats. Zona glomerulosa cells of control rats displayed 7-, 3·6- and 2-fold increases in the secretion of corticosterone, 18-hydroxycorticosterone and aldosterone; moreover, they released conspicuous

Table 1. *Plasma hormonal concentrations*

Experimental groups	Corticosterone (μg dl^{-1})	Aldosterone (ng dl^{-1})
Control rats	12·6 ± 4·3	24·6 ± 8·1
ACTH-treated rats	19·2 ± 7·5*	16·8 ± 5·4*

Values are group means ± S.D. * $P < 0{\cdot}01$ (Student's t test; n = 20).

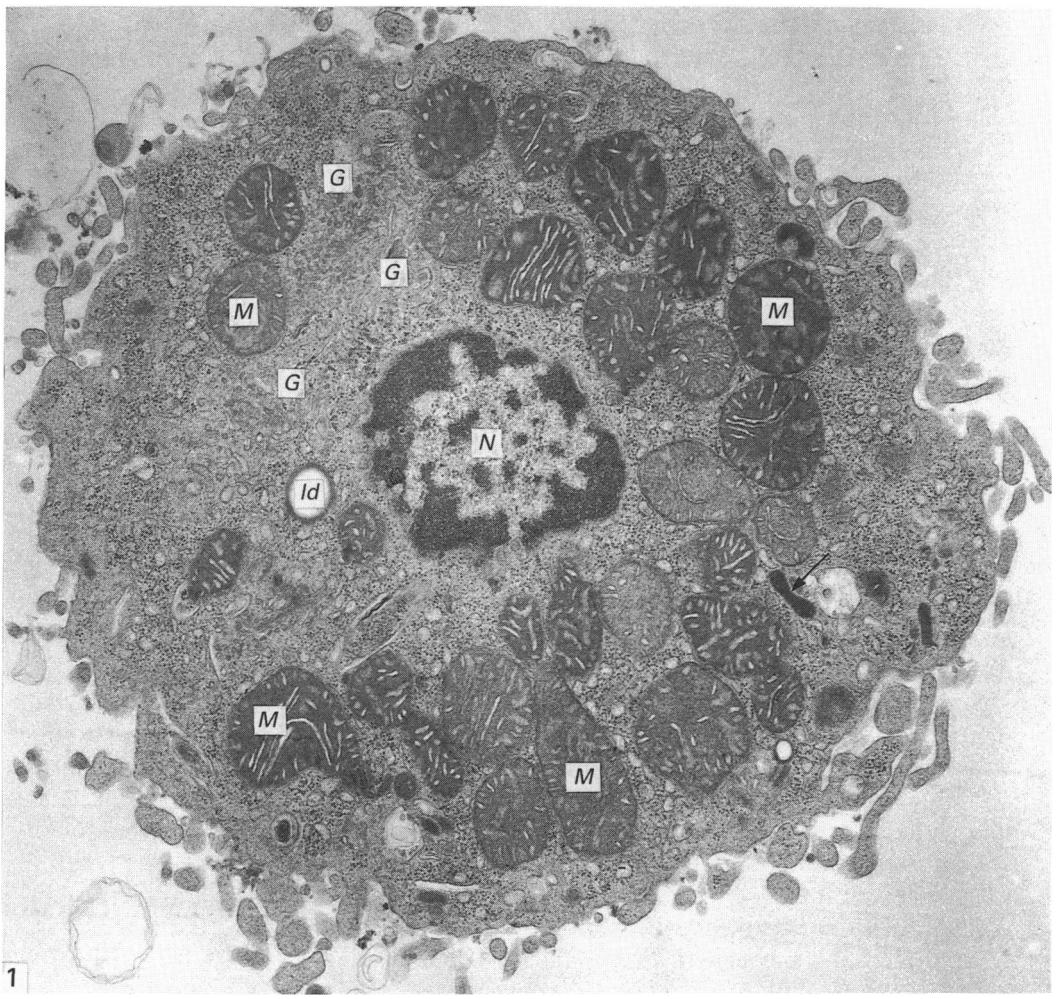

Fig. 1. Isolated zona glomerulosa cell of a control rat. Mitochondria (*M*) possess tubulo-lamellar cristae. Nucleus (*N*), Golgi apparatus (*G*), a small lipid droplet (*ld*) and a dense body (arrow) are indicated. × 19000.

amounts of 11-deoxycorticosterone and 18-hydroxy-11-deoxycorticosterone, while the secretion of progesterone underwent a 37% drop. Zona glomerulosa cells of ACTH-pretreated rats showed 5- and 2-fold rises respectively in their production of corticosterone and aldosterone. The release of 11-deoxycorticosterone and 18-hydroxycorticosterone was smaller than that of control rat ZG cells (−61 and −47%,

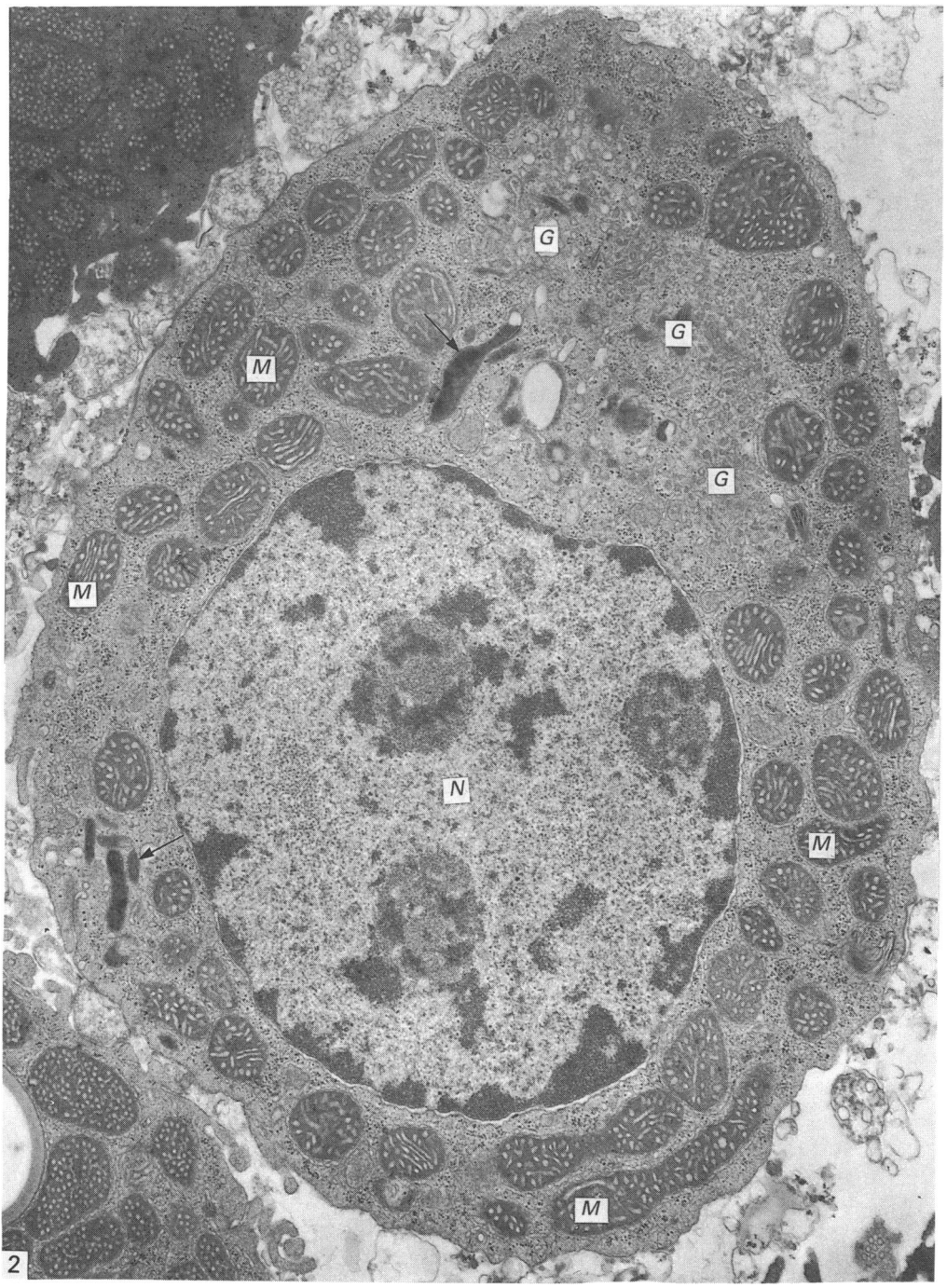

Fig. 2. Isolated zona glomerulosa cell of a rat pretreated with ACTH. Mitochondria (*M*) contain abundant tubulo-convolute cristae. Nucleus (*N*), Golgi apparatus (*G*) and dense bodies (arrows) are indicated. × 17500.

Table 2. *Morphometric parameters of isolated zona glomerulosa cells*

Experimental groups	Volume of cells (μm^3)	Volume of nuclei (μm^3)	Volume of mitochondrial compartment (μm^3/cell)	Surface area of mitochondrial cristae (μm^2/cell)
Control rats	532·8 ± 123·1	94·7 ± 15·6	134·2 ± 29·1	1852·8 ± 351·7
ACTH-treated rats	842·6 ± 214·2*	138·5 ± 18·4*	215·6 ± 38·5*	3537·5 ± 702·8*

	Volume of membrane space (μm^3/cell)	Surface area of agranular endoplasmic reticulum (μm^2/cell)	Volume of lipid droplet compartment (μm^3/cell)	Volume of dense body compartment (μm^3/cell)
Control rats	241·6 ± 50·6	2536·8 ± 580·2	59·6 ± 12·4	3·7 ± 1·0
ACTH-treated rats	454·0 ± 83·9*	5004·7 ± 924·8*	31·6 ± 10·3*	3·8 ± 1·2

Values are group means ± S.D. * $P < 0{\cdot}01$ (Student's t test; $n = 8$).

respectively), and 18-hydroxy-11-deoxycorticosterone was not secreted. No significant changes were observed in the release of progesterone.

DISCUSSION

According to previous investigations (see Introduction), prolonged ACTH treatment causes a significant reduction in the basal level of circulating aldosterone, whereas it notably enhances the growth of ZG cells in rats. Zona glomerulosa cell hypertrophy is mainly due to the increase in the volume of the mitochondrial compartment and to the proliferation of AER. Furthermore, the hypertrophy of the mitochondrial compartment is coupled with a striking proliferation of the mitochondrial inner membranes, whose morphological counterpart is the appearance of tubulo-convolute instead of tubulo-laminar cristae. This last finding is not completely in keeping with those reported by previous investigators, who described a complete fascicularisation of ZG cell mitochondria after chronic ACTH exposure (i.e. the transformation of tubulo-laminar cristae into a homogeneous population of vesicles) (McDougall *et al.* 1980; Nussdorfer *et al.* 1982; Mazzocchi *et al.* 1986). At present, we cannot reasonably explain this discrepancy, except by admitting differences in the preservation of mitochondrial morphology between isolated and *in situ* ZG cells.

It is well known that the enzymes of steroid synthesis are located in the mitochondrial cristae and AER membranes (Tamaoki, 1973; Nussdorfer, 1986), whose surface area per cell has been shown to be directly related to the activity per cell of some of these enzymes (Nussdorfer & Mazzocchi, 1983; Mazzocchi *et al.* 1986). However, the secretory activity of ZG cells from ACTH-pretreated rats, as evaluated by the overall production of post-pregnenolone steroids, seems to be notably decreased (basal secretion, −35%; stimulated secretion, −62%). These apparently conflicting findings could be explained by assuming that prolonged ACTH treatment depletes the ZG cell stores of steroid hormone precursors. The main precursor in steroid hormone synthesis is cholesterol, which rat adrenocortical cells take up from circulating high-density lipoproteins (Gwynne & Strauss, 1982; Boyd, McNamara, Suckling & Tocher, 1983). Exogenous cholesterol is stored in the lipid droplets as a reserve material (Moses, Davis, Rosenthal & Garren, 1969; Frühling *et al.* 1973; Nussdorfer, 1986). Thus, the net decrease in the volume of the lipid droplet compartment is in keeping

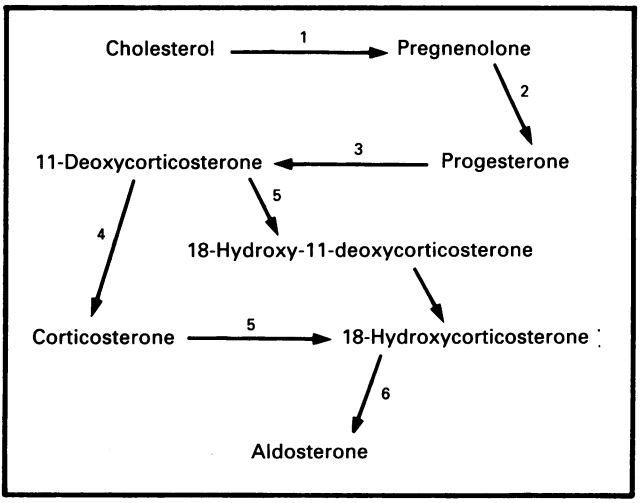

Fig. 3. Scheme illustrating the pathway of aldosterone synthesis in rats. 1, cholesterol side-chain cleaving enzymes; 2, 3β-hydroxysteroid dehydrogenase; 3, 21-hydroxylase; 4, 11β-hydroxylase; 5, 18-hydroxylase (monoxygenase I); 6, 18-dehydrogenase (monoxygenase II).

with our contention that five injections of a high dose of ACTH have depleted cholesterol stores in ZG cells, with consequent reduction especially of the stimulated steroid production.

The steroidogenic pathway in rat ZG cells is rather complex and is summarised in Figure 3. ACTH is known to activate acutely all the enzymes involved in aldosterone synthesis starting from progesterone (for review, see Nussdorfer, 1986). This may explain the secretion by ZG cells of control rats of some intermediate products (i.e. 11-deoxycorticosterone and 18-hydroxy-11-deoxycorticosterone), which are not released under basal conditions, as well as the rise in the rate of secretion of corticosterone, 18-hydroxycorticosterone and aldosterone and the reduction in the release of progesterone. The same occurs, though to a lesser extent (possibly due to the decreased availability of cholesterol, see above), in ZG cells from ACTH-pretreated rats. However, despite the reduction of overall steroid secretion, our HPLC data indicate that chronic ACTH pretreatment doubles both basal and stimulated production of aldosterone, the specific end-mineralocorticoid hormone secreted by ZG cells. This would suggest that ACTH not only acutely activates monoxigenase II, but is also able to induce an increased availability of this enzyme, when administered chronically, thus allowing a more complete transformation of newly formed 18-hydroxycorticosterone.

This result is rather surprising in view of the net drop in the plasma concentration of aldosterone in chronically ACTH-treated rats, reported in present and previous studies (see Introduction). On these grounds the current hypothesis that prolonged ACTH treatment induces not only morphological but also functional fascicularisation of ZG cells does not now seem tenable. The conspicuous rise in the basal blood level of corticosterone, the main glucocorticoid secreted in *Rodentia* (Sandor, Fazekas & Robinson, 1976), observed in ACTH-treated rats, must be exclusively ascribed to the chronic stimulation of inner adrenocortical layers and not to a shift of ZG cells from aldosterone to corticosterone production.

In the light of the present findings, it seems reasonable to hypothesise that a chronic exposure to high levels of ACTH provokes an enhancement in the metabolism of aldosterone rather than a decrease in its production. In the rat, the biliary route is the

Table 3. *Secretory activity of isolated zona glomerulosa cells*

Hormonal production (2×10^5 cells h^{-1})	Control rats		ACTH-treated rats	
	Basal	ACTH (10^{-8} M)	Basal	ACTH (10^{-8} M)
Progesterone	103·3 ± 42·1	65·1 ± 12·7†	52·5 ± 8.3[b]	59·8 ± 20·0
11-deoxycorticosterone	—	1084·2 ± 246·9	—	423.9 ± 177·0[b]
Corticosterone	114·6 ± 45·7	842·9 ± 94·1*	55·7 ± 28·6[b]	249·1 ± 18·6*·[b]
18-hydroxy-11-deoxycorticosterone	—	230·3 ± 39·6	—	—
18-hydroxycorticosterone	17·6 ± 8·9	62·9 ± 12·9*	—	40·0 ± 16·7[b]
Aldosterone	36·6 ± 15·9	77·1 ± 7·1*	69·3 ± 22·4[b]	124·1 ± 58·3†·[a]

Values are group means ± S.D. † $P < 0.05$ and * $P < 0.01$ versus basal secretion; [a] $P < 0.05$ and [b] $P < 0.01$ versus the respective control group (multiple range test of Duncan; n = 6).

major pathway of aldosterone excretion, the urinary route being a minor one (Morris, Silverman & Tsai, 1976). The rate of urinary excretion of aldosterone is directly related to the level of circulating hormone, so that it decreases sharply after prolonged ACTH treatment (Riondel *et al.* 1987). Studies on the hepatic metabolism of aldosterone in chronically ACTH-treated rats are required to gain insight on this point.

SUMMARY

Prolonged (5 day) treatment of rats with high doses of ACTH caused a significant reduction in the plasma concentration of aldosterone and a notable rise in that of corticosterone. Outer subcapsular (zona glomerulosa [ZG]) adrenocortical cells were isolated, and their morphology and secretory activity was investigated. ACTH pretreatment induced a marked hypertrophy of ZG cells which was coupled with significant increases in the volume of the mitochondrial compartment and in the surface area per cell of mitochondrial cristae and AER tubules, as well as with a striking lipid droplet depletion. Mitochondrial cristae were found to change from a tubulo-laminar to a tubulo-convolute configuration. Despite their hypertrophy, ZG cells from ACTH-pretreated rats displayed a conspicuous decrease in both basal and stimulated overall production of post-pregnenolone steroids, which was ascribed to the depletion of their stores of steroid hormone precursors (i.e. cholesterol and cholesterol esters contained in the lipid droplets). However, both basal and stimulated secretion of aldosterone was doubled, suggesting that chronic ACTH treatment induces in ZG cells an increased availability of monoxygenase II, the enzyme involved in the transformation of 18-hydroxycorticosterone into aldosterone. In the light of these findings, the drop in the plasma level of aldosterone observed in rats after prolonged treatment with ACTH is assumed to be due to an enhanced metabolism of aldosterone, possibly at the hepatic level.

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