
ULTRASTRUCTURAL AND BIOCHEMICAL EVIDENCE FOR
A STEROID-CONTAINING SECRETORY
ORGANELLE IN THE PERFUSED CAT ADRENAL GLAND

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Although it is generally agreed that the mitochondria and agranular endoplasmic reticulum play a central role in steroid hormone synthesis in the adrenal cortex (12), the histophysiological details of the sequence of events linking steroid synthesis and release are poorly understood (2, 12). In contrast to other hormones, which are sequestered in membrane-bounded organelles and are released by exocytosis (5, 14, 17), steroid hormone shows little or no storage, but in response to ACTH it appears to be synthesized and immediately released (7, 9). It has been argued that newly synthesized steroids leave the cell by simple diffusion (13), but this postulate cannot be rigorously evaluated owing to lack of adequate evidence. On the other hand, since a concomitant release of specific intragranular protein and hormone has been accepted as biochemical evidence for exocytosis in the adrenal medulla and neurohypophysis, for example (14, 17), the recent work from our laboratory showing that ACTH causes a temporally correlated release of protein and corticosteroid from the perfused cat adrenal gland (11, 15) provides evidence that the mechanism of steroid hormone secretion shares certain basic features with the secretory mechanism of other hormones. But before the question can be resolved as to whether steroids are extruded by a process akin to exocytosis, evidence must be provided for the existence of secretory inclusions in cortical cells. Although there is as yet no definitive evidence for steroid-containing granules in the adrenal cortex, a recent study of ovine luteal cells during the estrous cycle revealed a correlation between the morphologic demonstration of densely staining granules—which subcellular fractionation

studies showed were rich in progesterone—and the phasic pattern of progesterone secretion (3). In the present studies, ultrastructural and biochemical changes induced by ACTH in the perfused cat adrenal gland were investigated in order to seek additional evidence linking steroid release to the presence of intracellular secretory organelles.

MATERIALS AND METHODS

Adrenal glands of cats were perfused *in situ* at room temperature according to the method of Douglas and Rubin as previously described (1). In certain experiments both glands were perfused simultaneously, with the left gland used as the unstimulated control and the right gland exposed to ACTH after the left gland was removed (10). Samples of perfusate collected at 10-min intervals via a cannula placed in the adrenolumbar vein were assayed for corticosteroid (mainly as hydrocortisone) by an ethanolic sulfuric acid-fluorescence method (9). Adrenal glands removed immediately at the termination of a given experiment were analyzed for steroid as previously described (9).

For lysosomal enzyme determinations, perfused adrenal glands were removed, hemisected, and demedullated as completely as possible. The cortex was weighed, minced, and homogenized in a 0.25 M sucrose 0.01 M imidazole buffer (pH 7.2; wt/vol 1:10), and aliquots (50–200 μ l) of the homogenate were assayed for β -glucuronidase (4) and *n*-acetyl- β -glucosaminidase (19).

Eight adrenal glands were examined morphologically. Four were perfused with normal Locke's solution for 90 min, and four were perfused with Locke's solution for 50 min and with Locke's solution plus ACTH (100–250 μ U/ml) for an additional 40 min. The glands were then immediately fixed *in situ* by perfusion with glutaraldehyde-paraformaldehyde (3); strips of adrenal tissue were postfixed in buffered osmium tetroxide, dehydrated, and embedded in Araldite. The sections were stained se-

quentially with uranyl acetate and lead citrate before being examined in an Hitachi HU-11C electron microscope. Morphometric analysis of several sections was carried out at a magnification of 21,000 to evaluate the relative area occupied by certain cytoplasmic organelles with respect to total area (6).

β -1-24 ACTH was a generous gift from Ciba Pharmaceutical Company, Summit, N. J. All chemicals were of reagent grade and were purchased from commercial sources.

RESULTS

Cortical Cell Morphology

Despite prolonged perfusion of cat adrenals with saline medium, the ultrastructural characteristics of the fasciculata cells were well preserved. These cells were characterized by (a) a tubular network of agranular endoplasmic reticulum (Figs. 1, 2, and 4), (b) spherical mitochondria with tubular or vesicular cristae (Figs. 1 and 2) similar to those seen in fasciculata cells of other species (12), and (c) lipid droplets of fairly uniform size (Fig. 2). The Golgi regions were associated with vesicles containing electron-dense material (Figs. 2 and 4). These electron-dense membrane-bounded granules were predominantly found in ACTH-stimulated fasciculata cells, in close proximity to the plasma membrane (Fig. 5) and to the Golgi regions (Figs. 2-4). An increase in densely staining bodies (0.2-0.4 μ m; Figs. 3-5) was prominent in all cells of stimulated glands. Although granules were observed close to the cell membrane, no profiles demonstrating exocytosis were demonstrable; however, granules situated near microtubules could be found (Fig. 5).

To provide a more quantitative assessment of the effect of ACTH on the number of granules, an estimate of the relative percentage area occupied by the granules was determined in six representative areas from control and ACTH-stimulated glands (see Methods). ACTH treatment increased the granule relative volume from 1.3 to 5.4%—an increase of 4.2-fold (Fig 6a).

Adrenal Steroid Content

In addition to evoking a prolonged enhancement of steroid release (9), ACTH increased the steroid concentration of perfused adrenal glands from 6.0 to 37.4 nmol/g (Fig. 6b). When the steroid content of ACTH-stimulated glands was expressed as a percent of the steroid content of control glands in each experiment, ACTH in-

creased the steroid content by an average of 5.1-fold (± 0.2).

Cortical Lysosomal Enzyme Activity

Subcellular fractionation studies have provided evidence that β -glucuronidase is associated with lysosomes of the adrenal cortex (16). Homogenates of glands perfused for 40 min with ACTH showed no increase in the activity of this lysosomal enzyme (Fig. 6c). Similarly, ACTH failed to augment the activity of another lysosomal enzyme, *n*-acetyl- β -glucosaminidase. β -Glucuronidase and *n*-acetyl- β -glucosaminidase activities of ACTH-treated glands were 102 ± 6 and $101 \pm 7\%$, respectively, of the activities of corresponding control glands.

DISCUSSION

Cat adrenal glands perfused *in situ* for 60-90 min with Locke's solution secrete very low levels of corticosteroid (9). The addition of physiological concentrations of ACTH to the perfusion medium rapidly augments the steroid content of the gland; and this increased level of glandular steroid is maintained, despite large quantities of steroid being released (9). Morphological analysis of such control and stimulated glands clearly demonstrates that the ACTH-induced increase in intracellular and released steroid is accompanied by an increase in the number of certain cytoplasmic inclusions in cells of the zona fasciculata. These organelles are 0.2-0.4 μ m in diameter and appear to originate from the Golgi region. Similar structures have been previously identified in the adrenal cortex (2, 8, 18), and a greater number of such dense bodies have been observed in the fasciculata cells of the rat after exposure to ACTH (18). In the latter investigations, however, these granules were characterized as lysosomes on the basis of acid phosphatase activity. However, in the present study ACTH failed to augment cortical lysosomal enzyme activity; an increase in enzyme activity would be expected if ACTH stimulation were associated with a dramatic increase in the number of lysosomes.

Thus, serious consideration must be given to the possibility that these inclusions contain steroid and that they serve as the vehicle for the extrusion of hormone from the cell. This possibility is a cogent one in light of the striking fact that the fourfold increase in the number of these granules elicited by ACTH was matched by a fivefold increase in

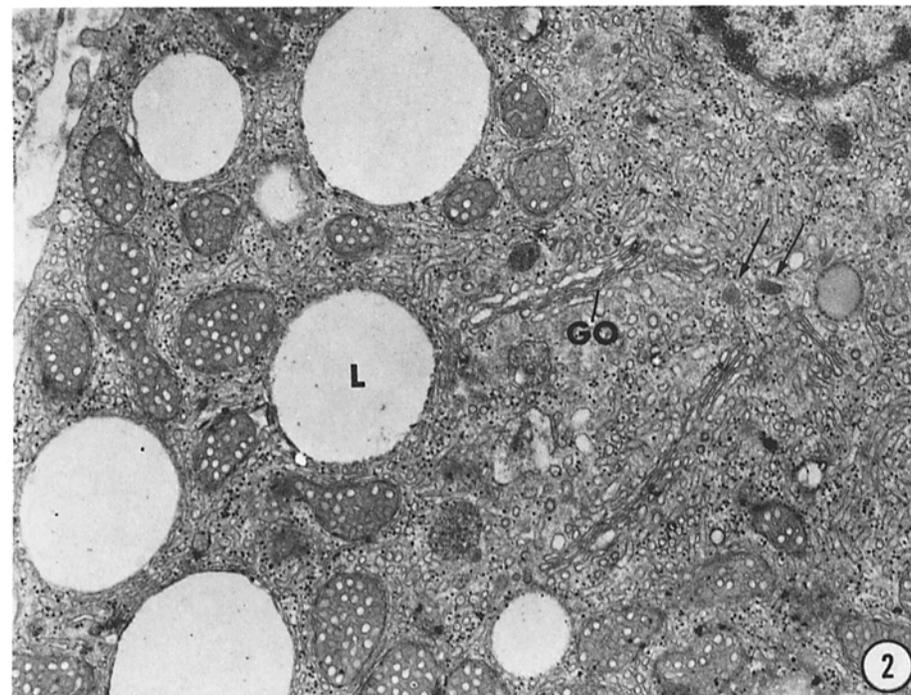
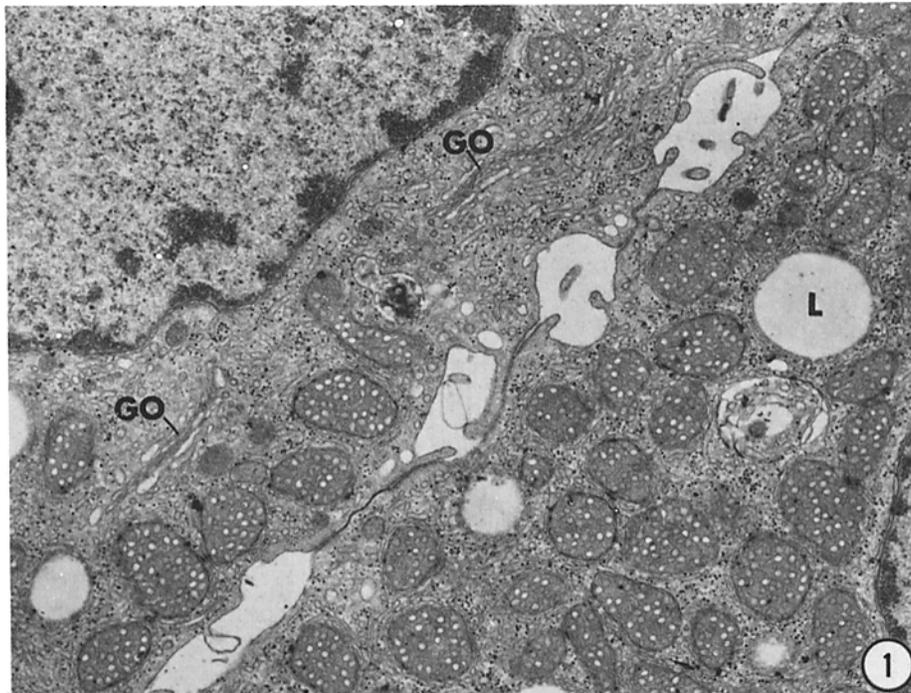


FIGURE 1 Portions of two fasciculata cells obtained from an adrenal gland perfused with Locke's solution for 90 min (steroid output 50 ng/min). Numerous spherical mitochondria and abundant agranular endoplasmic reticula are seen. Golgi regions (*GO*) and lipid droplets (*L*) are also prevalent. $\times 19,000$.

FIGURE 2 A similar area of the cell from the same adrenal as shown in Fig. 1. In addition to depicting lipid droplets (*L*) and numerous mitochondria, the micrograph shows small, moderately electron-dense granules (arrows) that appear to be associated with the Golgi region (*GO*). $\times 22,500$.

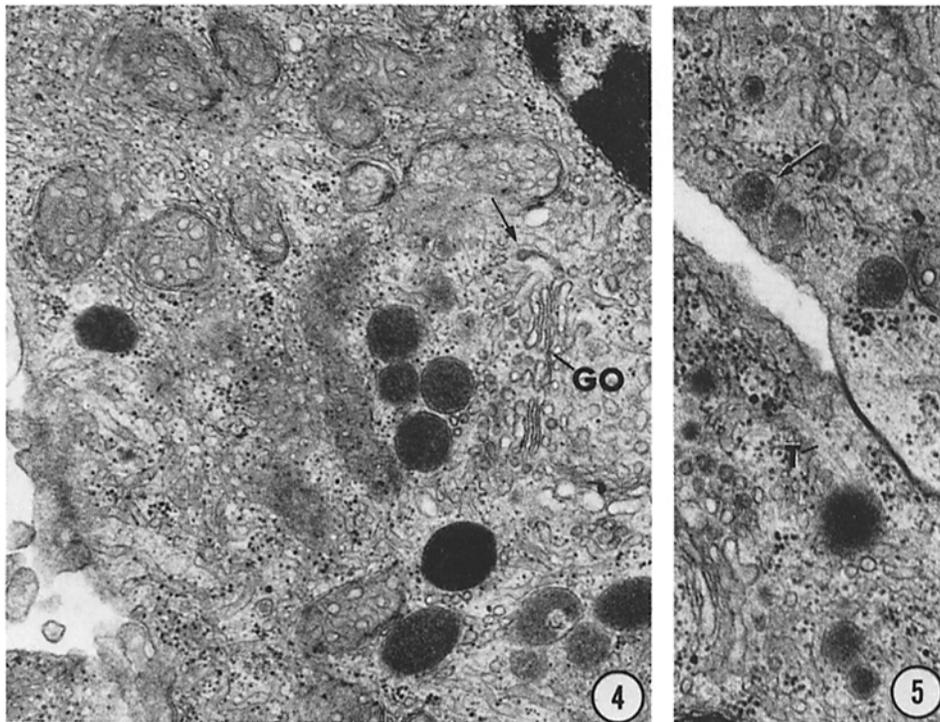
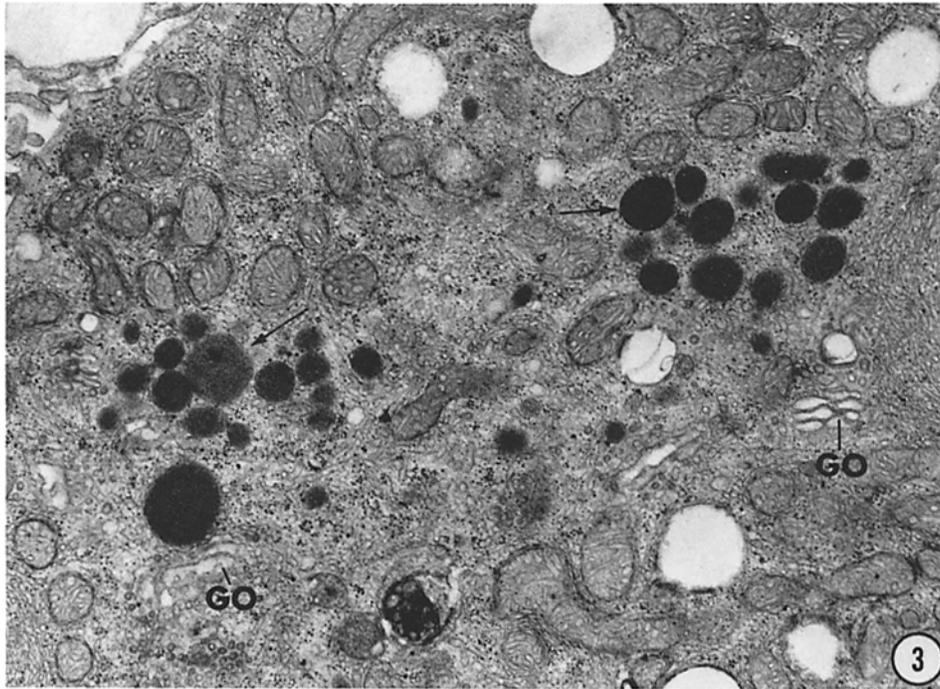


FIGURE 3 A region of the fasciculata cell obtained from an adrenal stimulated with ACTH for 40 min (steroid output 300 ng/min). Densely staining granules (arrows) are prevalent in areas of the cell in close proximity to the Golgi regions (GO). Cf. Figs. 1 and 2. $\times 22,500$.

FIGURE 4 Another cell from an ACTH-stimulated adrenal in which the dense-staining granule (arrow) appears to be originating in the Golgi region (GO). $\times 30,000$.

FIGURE 5 Preparation taken from an ACTH-stimulated gland which shows granules (arrow) in close proximity to the plasma membrane. A granule in close association with a microtubule (T) is also seen. $\times 30,000$.

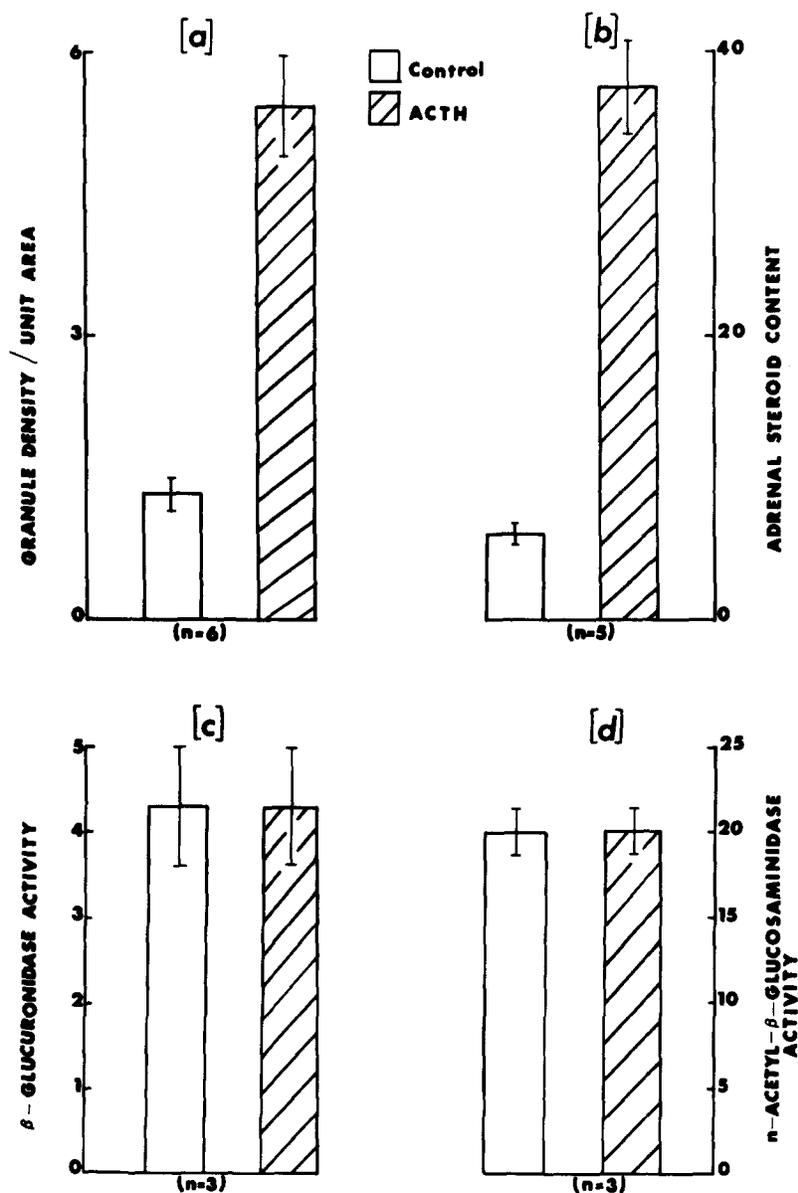


FIGURE 6 The effect of ACTH on (a) granule relative volume, (b) steroid concentration, and (c and d) lysosomal enzyme activity of perfused adrenal glands. Both adrenals were simultaneously perfused with Locke's solution. After 50 min of perfusion, the left gland was removed, and perfusion of the right gland was continued for an additional 40 min with ACTH (100–250 μ U/ml). Morphometric or biochemical analysis was then carried out as described in Materials and Methods. Each vertical bar represents mean values (\pm SEM). The number in parentheses indicates the number of glands employed for each determination. Steroid concentrations are expressed as nanomoles/gram wet weight of tissue; and β -glucuronidase and *n*-acetyl- β -glucosaminidase activities are expressed as micromoles/minute/gram protein.

the steroid content of the gland. Furthermore, previous studies from our laboratory on the perfused cat adrenal gland demonstrated that ACTH-induced steroid secretion can be temporally corre-

lated with protein release (15); and that this protein secretion, like steroid secretion, depends upon the presence of calcium (15). These biochemical studies support the view that steroid hor-

hormone is released by a process identical with—or related to—exocytosis (15).

Although the granules identified in the present study could be visualized in close proximity to the plasma membrane, there was no evidence of exocytotic figures, or of granules in the process of leaving the cells; this imparts a degree of uncertainty to the interpretation of our present findings. The relative paucity of these granules may, at least in part, account for the difficulty of visualizing them in an obvious state of secretory activity, especially since morphological demonstration of exocytosis is not a simple task even in the medullary chromaffin cell where large stores of catecholamine are invariably present (5).

Although, on the basis of the presently available evidence, it is still premature to affirm conclusively that the inclusions in the cortex induced by the action of ACTH are secretory organelles, the present investigation offers intriguing evidence that cannot be ignored if the mechanism of steroid release is to be elucidated ultimately. Additional studies will be pursued to provide more rigorous proof regarding the nature of these organelles which are under such exquisite control of actions of ACTH.

SUMMARY

A correlative study of the ultrastructural and biochemical effects of ACTH on fasciculata cells was carried out on the isolated cat adrenal gland perfused *in situ* with Locke's solution. The outstanding morphologic feature of cortical cells exposed to microunit ACTH concentrations for 40 min was the abundance of electron-dense granules (0.2–0.4 μm). These organelles were observed in small groups in close proximity to the Golgi region and to the cell membrane. Morphometric and biochemical analysis of control and ACTH-treated glands demonstrated that ACTH stimulation was associated with a fourfold increase in the number of these granules and a comparable increase in the corticosteroid content of the gland. By contrast, ACTH failed to augment cortical lysosomal enzyme activity. These findings, which link steroid release to the appearance of intracellular granules, extend further the parallels between the mechanism of release of newly synthesized steroid and the release of preformed hormones stored in secretory organelles. These results also lend support to the concept that a process related to exocytosis may be the underlying mechanism for extruding steroid from the cortical cell.

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