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IDENTIFICATION OF THE CYCLOHEXIMIDE-SENSITIVE SITE IN ANGIOTENSIN-STIMULATED ALDOSTERONE SYNTHESIS*

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Abstract—We have investigated the action of a protein synthesis inhibitor on the ability of angiotensin II (AII) to stimulate steroid synthesis. Isolated bovine adrenal glomerulosa cells were incubated in the presence and absence of angiotensin and cycloheximide, and the effects of the inhibitor on six cellular processes were measured. Cycloheximide at 7 and 28 μM inhibited the ability of the hormone to stimulate aldosterone synthesis. These concentrations of cycloheximide blocked protein synthesis by 72 and 79% respectively. Cycloheximide did not block receptor binding of angiotensin, the effect of angiotensin on [^{32}P]phosphate incorporation into phosphatidylinositol, nor the ability of the hormone to alter $^{45}Ca^{2+}$ fluxes. Mitochondrial conversion of cholesterol to pregnenolone is thought to be the rate-determining step in corticosteroid synthesis. Mitochondria isolated from cells treated with angiotensin made pregnenolone at a higher rate than control mitochondria. Cycloheximide blocked this effect when it was present in the cell incubation medium with angiotensin. Cycloheximide added directly to mitochondria had no effect on pregnenolone synthesis. Cycloheximide also blocked AII stimulation of pregnenolone synthesis in intact cells. We propose that protein synthesis is required for angiotensin to exert its stimulatory effects at one particular locus: activation of mitochondrial pregnenolone synthesis. Protein synthesis is not required for other angiotensin-stimulated processes in bovine adrenal glomerulosa cells.

Angiotensin II stimulates aldosterone synthesis *in vivo* and *in vitro*. The pathway for aldosterone synthesis is as follows: cholesterol → pregnenolone → progesterone → deoxycorticosterone → corticosterone → 18-hydroxycorticosterone → aldosterone [1–5]. The mechanism of action of angiotensin involves binding to plasma membrane receptors [6, 7] but is otherwise poorly defined. There is evidence to support roles for altered phospholipid metabolism [8–11] and calcium fluxes [10, 12–16], and at least permissive roles for potassium, protein synthesis, and arachidonic acid metabolism [17–22]. There is evidence that angiotensin treatment increases the activity of enzymes catalyzing cholesterol side-chain cleavage and later steps [23–25].

Inhibition of protein synthesis by cycloheximide and puromycin inhibits angiotensin-stimulated aldosterone synthesis [8, 10, 20–22] by an unknown mechanism. Protein synthesis inhibitors have played an important role in research on adrenocorticotropic hormone (ACTH) stimulation of steroidogenesis. The step that is most susceptible to cycloheximide inhibition is precisely the step which ACTH most acutely stimulates, the movement of cholesterol within the mitochondrion to a location accessible to cytochrome P-450 [26–32]. Neither the movement of cholesterol to mitochondria nor cholesterol side-chain cleavage itself is cycloheximide-sensitive [26, 28, 30]. The ability of cycloheximide to inhibit ACTH-stimulated steroid synthesis within a few minutes has led to the postulate of a "labile protein" required for ACTH action [33].

Much less is known about the mechanism of inhibition by cycloheximide of angiotensin stimulation of aldosterone synthesis. There is evidence to suggest that cycloheximide affects angiotensin-stimulated phospholipid metabolism [8] and the "late steps" from corticosterone to aldosterone [24].

We have developed methods for looking at six different processes in bovine adrenal glomerulosa cells: binding of [^{125}I]angiotensin; [$^{45}Ca^{2+}$]fluxes; [^{32}P]phosphate labeling of phosphatidylinositol; pregnenolone synthesis in cell suspensions and mitochondria; aldosterone synthesis; and protein synthesis. Work reported from Farese's laboratory [8, 9, 34, 35] has provided a stimulus for much of the work we report here.

MATERIALS AND METHODS

Reagents. The following materials were obtained: collagenase I (clostridium histolyticum collagenase, EC 3.4.24.3), Worthington Biochemical Corp. (Freehold, NJ); bovine serum albumin, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), sodium isocitrate, and diatomaceous earth (celite), Sigma Chemical Co. (St. Louis, MO); sucrose (density-gradient grade) and [4,5-^{3}H]leucine

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(15 Ci/mmole), Schwarz/Mann, division of Becton-Dickinson & Co. (Orangeburg, NY); pregnenolone antiserum, Radioassay Systems Laboratories, Inc. (Carson, CA); aminoglutethimide phosphate, a gift from the Ciba–Geigy Pharmaceutical Co. (Summit, NJ); trilostane, a gift from the Sterling Winthrop Research Institute (Rensselaer, NJ); angiotensin II (ile^5), Bachem Co. (Torrance, CA); polyethylene tubes for aldosterone radioimmunoassay, Sarstedt Co. (Princeton, NJ); [7-^{3}H]pregnenolone (10–25 Ci/mmole), [^{32}P]phosphate (285 Ci/mg) and $^{45}CaCl_2$ (4–30 Ci/g), New England Nuclear Corp. (Boston, MA); [^{125}I]aldosterone and aldosterone antiserum, Diagnostic Products Corp. (Los Angeles, CA); and isooctane, Burdick and Jackson Laboratories, Inc. (Muskegon, MI). All other compounds were reagent grade and obtained commercially.

Cell preparation. Adrenal glands from adult cows were obtained from the Oscar Mayer Co. (Madison, WI) or from the Champion Packing Co. (Waterloo, WI). Cells were prepared by collagenase digestion according to a previously published method [10, 12].

Measurement of aldosterone production from cell suspensions, binding of [^{125}I]angiotensin to cells, [^{32}P] phosphate labeling of phosphatidylinositol, and $^{45}Ca^{2+}$ influx experiments were performed as reported [10, 12].

Cell incubation and preparation of mitochondria. Cell incubations were performed in buffer (pH 7.4) containing 20 mM HEPES, 136.9 mM NaCl, 3.6 mM KCl, 1.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 11 mM glucose, 0.1% bovine serum albumin, and 0.76 mM aminoglutethimide or aminoglutethimide phosphate [36, 37]; pH 7.4. Cells were pipetted into 1.4 × 10 cm cellulose acetate tubes (approximately 20×10^6 cells/tube). Aminoglutethimide or aminoglutethimide phosphate was added, followed by angiotensin or other reagents. Final incubation volume was 2.0 ml. Tubes were incubated in a 37° shaking water bath for 45 min. Tubes were placed on ice, and to each tube was added 3 ml hypotonic buffer containing 10 mM Tris, 10 mM KCl, and 0.5 mM EDTA [38]. Tubes were centrifuged at 260 *g* for 3 min, the supernatant fractions were aspirated, and the cells were resuspended in 5 ml hypotonic buffer. Centrifugation and resuspension of cells were repeated three times. The final cell pellet was resuspended in 5 ml hypotonic buffer, and the cells were allowed to swell for 10 min. Cells were broken by 60 passes with a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 700 *g* for 10 min. The pellet was discarded and mitochondria were obtained by centrifuging the supernatant fraction at 10,000 *g* for 15 min. Mitochondria were resuspended in 0.1 M Tris–0.25 M sucrose, pH 7.4, at a protein concentration of 0.3 to 1.2 mg/ml. This preparation of adrenal mitochondria was based on published methods [38]. Cell incubations were performed in triplicate, with one mitochondrial preparation from each cell incubation tube.

Synthesis of pregnenolone. Incubations were performed in 1 × 7.5 cm polypropylene tubes in a final volume of 1 ml. Each tube received 0.2 to 0.5 ml of mitochondrial suspension, other reagents as indicated in 0.1 M Tris–0.25 M sucrose, pH 7.4, and 0.5 ml of mitochondrial assay mixture. The mitochondrial assay mixture contained 0.1 M Tris, 0.0176 M $MgCl_2$, 0.098 M KCl, 0.044 M K_2HPO_4, 0.2% bovine serum albumin, and 20 mM sodium isocitrate, pH 7.4 [39]. $MgCl_2$, albumin, and isocitrate were added to the mixture, and the pH was adjusted immediately before use.

Tubes were incubated in a 30° shaking water bath for varying time intervals, usually 10 or 15 min. The reaction was stopped by placing the tubes in an ice bath, adding 0.1 ml of 1.1 mM aminoglutethimide phosphate, and immediately centrifuging at 27,000 *g* for 30 min. Supernatant fractions were stored at −20° until assayed for pregnenolone.

Pregnenolone synthesis in intact cells was measured by incubating cells in the presence of trilostane, which blocks conversion of pregnenolone to progesterone [25, 40]. Except for the presence of this reagent, cells were incubated as described for measuremement of aldosterone production [10].

Radioimmunoassay for pregnenolone. Radioimmunoassay was performed directly on supernatant fractions [37, 41] according to published methods [42]. [7-^{3}H]Pregnenolone was purified before use on celite columns [43]. The sensitivity of the radioimmunoassay was 0.025 to 0.1 ng. The standard curve for pregnenolone was linear from 0.1 to 5.0 ng. The intra-assay coefficient of variation was 5.8%.

Protein determination. The protein content of mitochondrial suspensions was measured according to the method of Lowry *et al.* [44], using bovine serum albumin as a standard.

Protein synthesis. Protein synthesis was measured by incorporation of [^{3}H]leucine into acid-precipitable material according to published methods [45]. Cells (0.8 ml, 8.3 million per tube) were added to 1.2 × 6.4 cm cellulose acetate tubes on ice, 0.1 ml buffer or cycloheximide was added, followed by 0.1 ml [^{3}H]leucine (0.03 μCi). Tubes were incubated at 37°. Duplicate aliquots (0.2 ml) were removed at 15, 30, and 45 min and pipetted into tubes containing 1.0 ml of ice-cold 10% trichloroacetic acid. Zero-time aliquots were taken immediately after [^{3}H]leucine was added, while the tubes were still on ice. Tubes were centrifuged at 1100 *g* for 2 min, the supernatant fraction was aspirated and the pellet was resuspended in 1 ml of cold 5% trichloroacetic acid, and the tubes were again centrifuged. Pellets were transferred to counting vials with 1.0 ml water, 4 ml Bray's scintillation fluid was added, and vials were counted in a Searle Isocap 300 liquid scintillation counter.

RESULTS

As shown in Fig. 1, angiotensin increased aldosterone synthesis from bovine adrenal glomerulosa cells. When cycloheximide was added, no increase in aldosterone synthesis occurred. Binding of [^{125}I]angiotensin II to glomerulosa cell receptors was not inhibited significantly by 10 or 50 μM cycloheximide. In control experiments, a 1-hr incubation with cycloheximide (28 μM) did not affect cell viability as monitored by trypan blue exclusion. Moreover, cells incubated with cycloheximide (28 μM) and then washed free of inhibitor responded to angiotensin

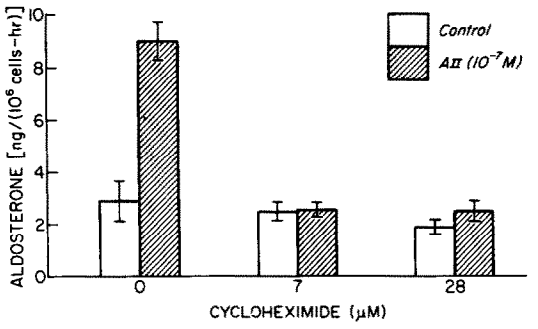

Fig. 1. Effect of cycloheximide on basal and angiotensin-stimulated aldosterone synthesis. Buffer, angiotensin and cycloheximide were added to cells as indicated and tubes were incubated for 45 min at 37°. Aldosterone present in the supernatant fraction at the end of the incubation was determined by RIA. Each bar is the mean ± S.E.M. of four determinations.

with production of aldosterone indistinguishable from untreated cells.

Figure 2 illustrates the uptake of [^{3}H]leucine into acid-precipitable material when glomerulosa cells were incubated with various levels of cycloheximide for 15, 30, and 45 min. Cycloheximide at 7 μM, a concentration which completely inhibits the effect of angiotensin on steroid synthesis, was as effective as higher levels as an inhibitor of protein synthesis. For all levels of drug tested, there was some residual protein synthesis.

Figure 3 shows the incorporation of [^{32}P]phosphate into phosphatidylinositol in the presence and absence of angiotensin and cycloheximide. Angiotensin stimulated phosphatidylinositol labeling, and cycloheximide at 7 and 28 μM did not block the effect of the hormone.

Another process that may be related to angiotensin-stimulated steroidogenesis is the effect of the hormone on calcium fluxes. Previous work from this

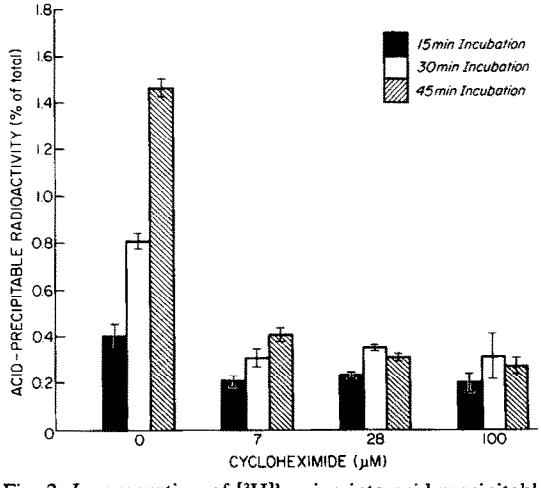

Fig. 2. Incorporation of [^{3}H]leucine into acid-precipitable material when glomerulosa cells were incubated at 15, 30, and 45 min at 37° with various levels of cycloheximide. Zero-time values (acid-precipitable radioactivity present with no incubation) were subtracted from the values shown. Each bar shows the mean and range for two determinations.

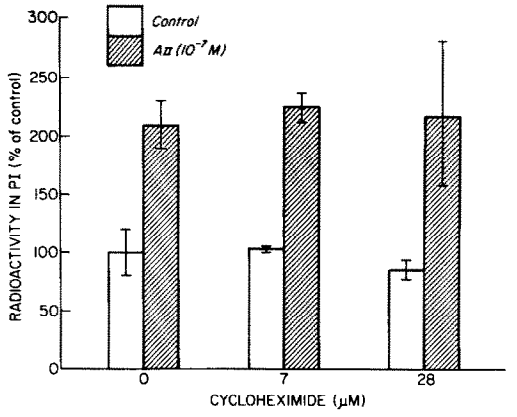

Fig. 3. Incorporation of $^{32}PO_4$ into phosphatidylinositol in the presence and absence of angiotensin and two doses of cycloheximide. Buffer, angiotensin, cycloheximide and $^{32}PO_4$ were added to cells as indicated, and tubes were incubated for 45 min at 37°. Phospholipids were extracted and [^{32}P]phosphatidylinositol was determined as described previously [10]. For this and following figures, each bar represents the mean ± S.E.M. from three incubation tubes.

laboratory [10, 12] has indicated that angiotensin causes $^{45}Ca^{2+}$ efflux from, and slows $^{45}Ca^{2+}$ influx into, bovine adrenal glomerulosa cells. Figure 4 shows the effect of angiotensin on $^{45}Ca^{2+}$ influx with and without cycloheximide. The influence of the hormone on $^{45}Ca^{2+}$ was unaffected by cycloheximide.

The results reported above indicate that, although angiotensin-stimulated steroid synthesis is inhibited completely at low doses of cycloheximide, other processes affected by the hormone are not. To define the locus at which cycloheximide exerted its inhibition, we measured mitochondrial pregnenolone synthesis. We incubated bovine adrenal glomerulosa cell suspensions in the presence of aminoglutethimide with and without angiotensin and measured the ability of mitochondria isolated from these cells to synthesize pregnenolone from endogenous cholesterol. Aminoglutethimide was added to the intact

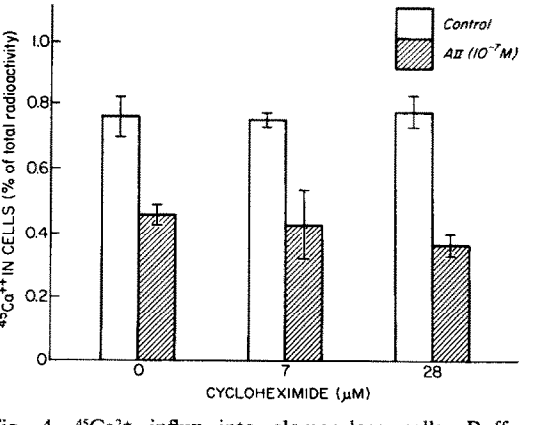

Fig. 4. $^{45}Ca^{2+}$ influx into glomerulosa cells. Buffer, cycloheximide, angiotensin and $^{45}Ca^{2+}$ were added to cells as indicated. Each incubation tube contained 1.6×10^6 cells and 1.0 μCi $^{45}Ca^{2+}$ in a final volume of 0.4 ml. $^{45}Ca^{2+}$ uptake into cells during a 45-min incubation at 37° was determined on 0.05-ml aliquots as described previously [12].

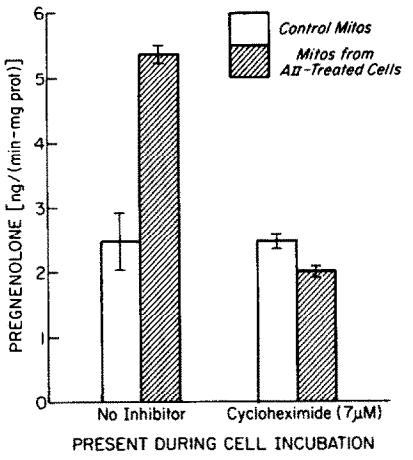

Fig. 5. Pregnenolone formation by mitochondria from cells treated with buffer or cycloheximide in the presence and absence of 10^{-7} M angiotensin. Cells were incubated for 45 min at 37°. Mitochondria were prepared and incubated with 10 mM isocitrate at 30° for 10 min as described. Pregnenolone in the supernatant fraction was determined by RIA.

cells because it inhibits cholesterol side-chain cleavage but allows the accumulation of cholesterol at a location accessible to the side-chain cleavage enzyme [27, 36, 46, 47]. Without this reagent, no stimulation by angiotensin II was observed in mitochondria. Mitochondria from hormone-treated cells synthesized on the average 2.2 times more pregnenolone than control mitochondria. As Fig. 5 shows, this effect was blocked by the addition of cycloheximide to the cell preincubation. Direct addition of 7 μM cycloheximide to mitochondria did not diminish pregnenolone synthesis (data not shown).

Figure 6 shows the results of an experiment in which aldosterone synthesis and pregnenolone synthesis were measured in separate incubation tubes in response to angiotensin in the presence and absence of cycloheximide. In the presence of trilostane, pregnenolone accumulates because it is not converted to progesterone or other steroids. Cycloheximide had similar inhibitory effects on angiotensin stimulation of aldosterone and pregnenolone synthesis.

It should be noted that, in the experiment depicted in Fig 6, cycloheximide inhibited basal steroid production. Inhibitory effects of cycloheximide on basal aldosterone production were quite variable, ranging from 7 to 74% (mean 47%), whereas angiotensin stimulation of aldosterone production was inhibited more uniformly and markedly, from 81 to 100% (mean 93%), for doses of cycloheximide ranging from 0.7 to 28 μM. Inhibition of basal aldosterone has been reported [8], but the reason for the variation we observed is not known.

DISCUSSION

The experiments reported here were performed under similar conditions so that the effects of cycloheximide on all the measured processes could be compared. All cell incubations were performed at 37° for 45 min in the same buffer and at the same cycloheximide levels. Our data indicate that the inhibitory action of cycloheximide on angiotensin action did not take place at the level of phosphatidylinositol labeling. This agrees with the work of Hunyady *et al.* [11]. Work from that laboratory and from Farese's laboratory [8] indicate that angiotensin stimulates [^{32}P]phosphate labeling of phosphatidylinositol by increasing the turnover (breakdown and synthesis) of this lipid. Our cycloheximide data and that of Hunyady *et al.* contrast with that of Farese, who has reported that this compound blocks angiotensin-stimulated labeling of phosphatidylinositol by [^{32}P]phosphate [8]. The experiments we report here shed no light on the mechanism of angiotensin-stimulated phosphatidylinositol labeling, but show that this effect is not blocked by cycloheximide. We show that cycloheximide at low doses dramatically and

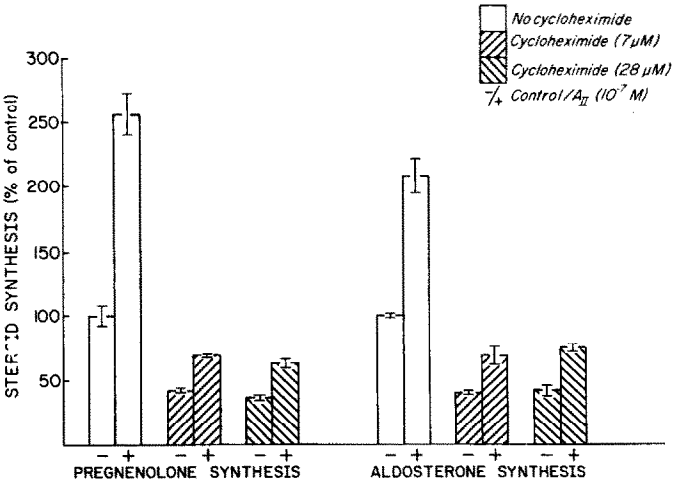

Fig. 6. Pregnenolone and aldosterone synthesis by cells in the presence or absence of 10^{-7} M AII with cycloheximide at 0, 7 or 28 μM. Cells were incubated for 45 min at 37°. When pregnenolone was measured, cells were incubated with trilostane (10 μM). Steroids in the supernatant fraction were determined by RIA.

specifically inhibited angiotensin-stimulated aldosterone and pregnenolone synthesis and stimulation of mitochondria. Low doses of cycloheximide did not affect either basal levels of calcium influx or phosphatidylinositol metabolism, or the effects of angiotensin on these processes. We suggest that the inhibitory effect of cycloheximide on angiotensin-stimulated steroid synthesis does not lie in its ability to affect receptors, phosphatidylinositol metabolism, or calcium fluxes.

Our results with angiotensin and mitochondria are very similar to those observed with adrenal mitochondria from rats treated with ACTH or from Y-1 adrenal cells treated with dibutyryl cyclic AMP: agonists "activate" mitochondria to make more pregnenolone and inhibition of protein synthesis blocks this effect [26–32, 48]. ACTH can stimulate cholesterol side-chain cleavage in at least two ways. First, it can increase mitochondrial cholesterol levels by the uptake of free cholesterol from plasma [49–51], and by hydrolysis of cholesterol esters and subsequent uptake of free cholesterol by mitochondria [28, 47, 52–54]. Cycloheximide does not block these effects of ACTH [26, 27, 47, 51]. The second way in which ACTH increases side-chain cleavage involves the movement of cholesterol within mitochondria, possibly from the outer to the inner mitochondrial membrane, where the cholesterol is accessible to the side-chain cleavage enzyme [26, 28, 30, 47, 55]. This does not necessarily involve increased levels of mitochondrial cholesterol, but rather the disposition of cholesterol within mitochondria. This step is inhibited by cycloheximide.

Although the mechanism by which angiotensin stimulates steroid synthesis is not understood in as much detail as that of ACTH, there are striking similarities between the effects of the two hormones: (1) the major acute effect of both hormones is to increase the rate of conversion of cholesterol to pregnenolone [5, 22, 24, 28, 29, 37]; (2) ACTH stimulation of steroid synthesis and angiotensin stimulation of aldosterone synthesis are inhibited by protein synthesis inhibitors [8, 10, 20–22, 26, 28, 33]; and (3) mitochondria from ACTH- or angiotensin-treated cells or animals make more pregnenolone than control mitochondria [24, 27, 30, 56]. The results we present here suggest a further similarity between the two hormones: cycloheximide blocks the ability of angiotensin as well as ACTH to increase the rate of mitochondrial cholesterol side-chain cleavage.

One laboratory has reported that mitochondria from rats treated *in vivo* with angiotensin for 5 min or 4 days demonstrate increased pregnenolone formation and that this effect is not blocked by cycloheximide [24]. Cholesterol ester hydrolase activity was measured in the acutely treated adrenals, and no effect of angiotensin on this enzyme was observed. Mitochondrial cholesterol levels were not measured, however, and as the authors pointed out, the increase they observed in mitochondrial cholesterol side-chain cleavage could have resulted from increased mitochondrial cholesterol levels in the hormone-treated rats. Aguilera *et al.* [23] showed that angiotensin administration or elevated endogenous levels of angiotensin can increase mitochondrial cholesterol and that this increase correlates well with increased mitochondrial pregnenolone synthesis.

By contrast with ACTH in fasciculata, angiotensin apparently does not elevate levels of cAMP in adrenal glomerulosa [57]. Therefore, although angiotensin may not use the same second messenger as ACTH, the two hormones exhibit strikingly similar mechanisms of action on steroidogenesis in mitochondria of their respective adrenal targets.

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