Comparative Effects of Angiotensin and ACTH on Cyclic AMP and Steroidogenesis in Isolated Bovine Adrenal Cells

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ABSTRACT The comparative effects of angiotensin II and adrenocorticotropic hormone (ACTH) on cyclic AMP and steroidogenesis were investigated employing isolated bovine adrenal cells from the zona fasciculata. Like ACTH, angiotensin produced a prompt increase in cyclic AMP which preceded the increase in corticosteroid production. Although this increase in cyclic AMP was small when compared to that induced by ACTH, it correlated with the amount of steroidogenesis. This observation is consistent with the view that cyclic AMP is the intracellular mediator of the steroidogenic action of angiotensin.

Angiotensin acted synergistically with ACTH on cyclic AMP levels. This synergism was not explained by inhibition of phosphodiesterase activity. Unlike ACTH, angiotensin failed to stimulate adenylate cyclase in broken cell preparations. The observations suggest that more than one mechanism may be involved in effects of ACTH and angiotensin on cyclic AMP levels.

INTRODUCTION

Angiotensin II and adrenocorticotropic hormone (ACTH)[1] are two polypeptide hormones known to stimulate adrenocortical steroidogenesis. ACTH has been extensively investigated and found to stimulate steroidogenesis through activation of the adenylate cyclase system (1, 2); however, relatively little attention has been focused on the mechanism of action of angiotensin on steroid biosynthesis. Angiotensin has been shown to induce aldosterone synthesis in man (3, 4) and in sheep with adrenal transplants (5), and to stimulate cortisol as well as aldosterone synthesis in isolated bovine adrenals (6, 7) and in the hypophysectomized dog (8, 9). However, attempts to demonstrate an effect of angiotensin on adrenal adenylate cyclase activity (10, 11) or cyclic AMP levels (12) have thus far been unsuccessful.

In the present study, we have compared the effects of angiotensin and ACTH on adrenal cyclic AMP and steroidogenesis in vitro, utilizing isolated cells from the bovine zona fasciculata. Our results demonstrate that angiotensin increases cyclic AMP levels in cells from the zona fasciculata, and they are consistent with the view that the steroidogenic action of angiotensin in fasciculata cells is mediated through cyclic AMP.

METHODS

Materials. Lot H of the United States Pharmacopeia corticotropin Reference Standard was used throughout this study. In order to express the doses of ACTH on a molar basis it was assumed that each ampule of the standard contained 1.5 i.v. U (13, 14) and that each unit of activity was equivalent to 10 μg of ACTH (2×10^{-9} mol) (15). Asparaginyl[1]-valyl[5] angiotensin II was purchased from Ciba Corp., Summit, N. J. 1 μg is equivalent to 10^{-9} mol. Appropriate dilutions of angiotensin and ACTH were made in acid-albumin-saline (0.6 ml 12N HCl, 1 g bovine albumin, and 8.5 g NaCl/liter of water). Twice crystallized trypsin and bovine serum albumin (BSA) (Cohn fraction V) were obtained from Sigma Chemical Co., St. Louis, Mo. Lima bean trypsin inhibitor was from Worthington Biochemical Corp., Freehold, N. J. 1-Methyl-3-isobutylxanthine (SC 2964) was kindly provided by the G. D. Searle

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[1] *Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; KRBG, Krebs-Ringer bicarbonate buffer; MIX, 1-methyl-3-isobutylxanthine.

and Co., Chicago, Ill. Tritiated cyclic AMP (SA-24 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Krebs Ringer bicarbonate buffer containing 200 mg glucose/100 ml (KRBG) was used to prepare the cells; for the incubation of the samples, albumin, 500 mg/100 ml, was added.

Methods. Isolated adrenal cells were prepared according to the method of Kloppenborg, Island, Liddle, Michelakis, and Nicholson (16) as modified by Sayers, Swallow, and Giordano (17). Eight calf adrenals were obtained from a local slaughter house, kept on ice in KRBG containing 500 mg albumin/100 ml until they were sliced with a Stadie-Riggs microtome delivering slices of 0.5 mm. The first (outer) slices, representing the glomerulosa and some fasciculata, were used for experiments reported elsewhere. The second or third slices, representing mainly fasciculata, were used for the present experiments. Slight contamination of the fasciculata preparation with glomerulosa cells was apparent from the fact that a given number of fasciculata cells contained about 10% as much aldosterone as would be contained in a similar number of cells prepared from the zona glomerulosa (outer 0.5 mm) of the calf adrenal. 2 g of slices were transferred to a cold (4°C) siliconized 125 ml Erlenmeyer flask containing 40 ml of KRBG and 250 mg trypsin/100 ml and stirred with a siliconized glass rod with a paddle-shaped end for 20 min in an atmosphere of 95% O_2 and 5% CO_2. The supernatant fluid, containing the cells, was then transferred to a plastic 250 ml Erlenmeyer flask kept on ice. This procedure was repeated 5 times, and the supernates were pooled and centrifuged at $100 \times g$ for 30 min at 4°C. The supernate was discarded and the sediment resuspended in 60 ml of KRBG containing 100 mg lima bean trypsin inhibitor/100 ml and 500 mg albumin/100 ml. After a second centrifugation at $100 \times g$ for 30 min the pellet was resuspended in approximately 60 ml of KRBG with 500 mg albumin/100 ml. Samples of 0.9 ml of this cell suspension, containing approximately 20 mg of cells, were distributed with a plastic pipet into polyethylene test tubes to which the appropriate additions of hormones were made in 0.1 ml acid-albumin-saline. The samples were incubated in a shaking water bath under an atmosphere of 95% O_2 and 5% CO_2. At the end of the incubation, the samples for steroid analysis were frozen until assayed, and samples for cyclic AMP determination were processed as described below.

Cyclic AMP determination. The samples (cells plus medium) were immediately homogenized in 0.3 N perchloric acid with tritiated cyclic AMP (3,000–4,000 cpm) to monitor for recovery. After centrifugation at $6,000 \times g$ for 15 min at 4°C, the supernate was applied to a 10×0.6 cm column of Dowex 50-X8 resin (100–200 mesh, in the hydrogen form) and eluted with 0.1 N HCl. The fraction containing cyclic AMP was lyophylized, reconstituted in 40 mM Na-acetate buffer (pH 4.0), and assayed for cyclic AMP by the method of Gilman (18). The apparent cyclic AMP content of extracts from incubations under different conditions was independent of the size of the sample assayed, and was destroyed by phosphodiesterase. Known amounts of cyclic AMP added to the samples could be accurately assayed. Perchloric acid did not affect the purification and/or assay for cyclic AMP.

Phosphodiesterase assay. Isolated bovine adrenal cells from the fasciculata were homogenized in 0.04 M Tris-HCl, pH, 7.5, with a Teflon glass homogenizer. The $2,000 \times g$ supernate and $2,000 \times g$ particles were assayed for phosphodiesterase activity as described by Beavo, Hardman, and Sutherland (19).

Adenylate cyclase assay. Cells from the fasciculata were homogenized in 1 mM potassium bicarbonate buffer (pH 7.4) with a Teflon glass homogenizer. At 4°C the homogenate was centrifuged at $2,000 \times g$ for 10 min, the sediment was washed twice, resuspended in the same buffer and used for the adenylate cyclase assay. The assay was based on the determination of the amount of cyclic AMP formed during 10 min of incubation at 37°C in the following medium: 4 mM ATP, 36 mM Tris-HCL, pH 7.4, 8 mM $MgSO_4$, 1 mM 1-methyl-3-isobutylxanthine (MIX), 0.2% albumin, and 0.01 mM $CaCl_2$. MIX is a phosphodiesterase inhibitor possessing about 10 times the potency of theophylline (20). Previous studies have shown that the activation of adrenal adenylate cyclase by ACTH requires the presence of calcium ions (21). The final volume was 0.5 ml. The reaction was stopped by the addition of perchloric acid in a final concentration of 0.3 N and by placing the samples in a boiling water bath for 3 min. Tritiated cyclic AMP (3,000–4,000 cpm) to monitor for recovery was added at the end of the incubation. The supernate was purified and assayed for cyclic AMP as described above.

Corticosteroid determination. The corticosteroids in the whole sample (cells plus medium) were measured as fluorogenic corticosteroids by the method of Mattingly (22). Further characterization of the fluorogenic corticosteroids

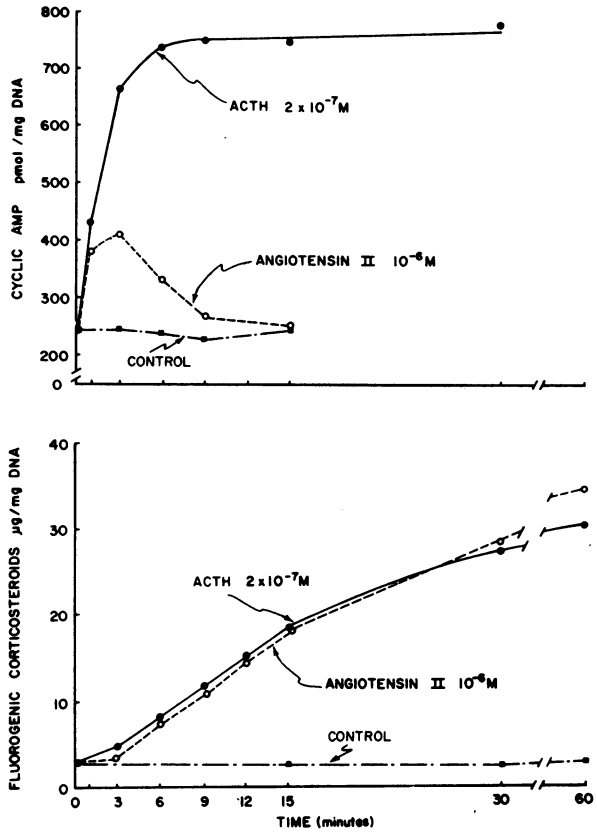

FIGURE 1 Upper panel: time-course of cyclic AMP accumulation in cells plus medium in response to angiotensin or ACTH.

Lower panel: time-course of fluorogenic corticosteroid accumulation in cells plus medium in response to angiotensin or ACTH. Each point represents the mean of two samples.

was accomplished by thin-layer chromatography using a dichloromethane: methanol (150:20) system. The areas corresponding to cortisol and corticosterone were eluted with 20% acetone in methanol and assayed fluorometrically. Procedural losses were monitored by additions of tritium-labeled corticosterone and carbon-14-labeled cortisol.

DNA and protein determination. DNA was measured according to the colorimetric method of Burton (23). Protein was measured by the method of Lowry, Rosenbrough, Farr, and Randall (24).

RESULTS

Time-course of cyclic AMP accumulation in response to angiotensin and ACTH. When bovine fasciculata cells were incubated in the presence of 2×10^{-7} M ACTH, cyclic AMP levels increased within 1 min, reached a peak within 6 min and remained elevated for 30 min (Fig. 1, upper panel). Angiotensin 10^{-6} M also produced a prompt increase in cyclic AMP which peaked at 3 min. However, the response to angiotensin was smaller and more transient than the response to ACTH, with cyclic AMP levels returning to control values within 9–15 min. In both cases, the doses of hormones used in this experiment were sufficient to stimulate maximal steroid production.

Time-course of corticosteroid accumulation in response

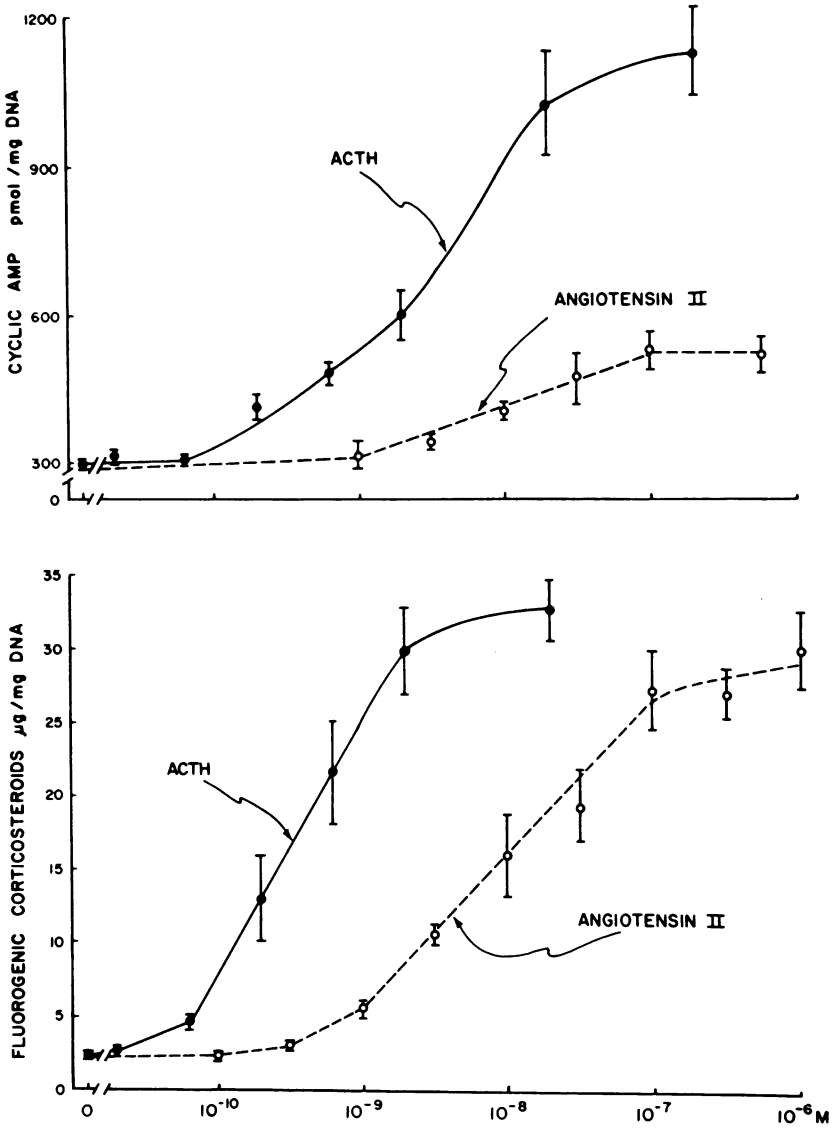

FIGURE 2 Upper panel: dose-response curve for angiotensin or ACTH on cyclic AMP accumulation in cells plus medium during 3 min incubation. $N = 4$. In these and subsequent experiments I = SEM.

Lower panel: dose-response curve for angiotensin or ACTH on fluorogenic corticosteroid accumulation in cells plus medium during 60 min incubation. $N = 4$.

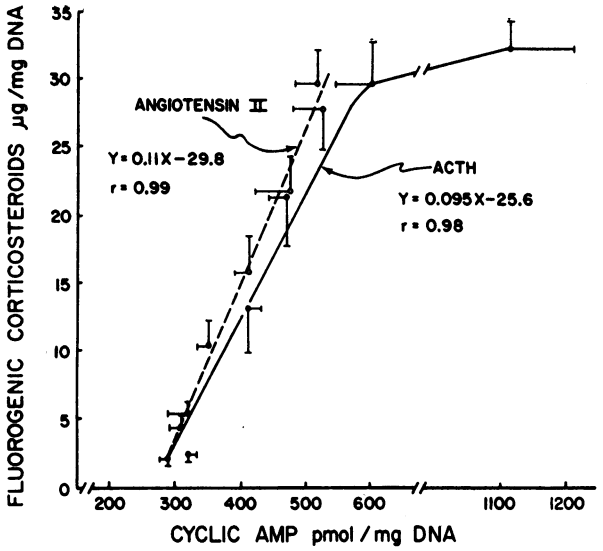

FIGURE 3 Correlation between cyclic AMP and fluorogenic corticosteroids in response to angiotensin or ACTH. Samples for cyclic AMP determination were incubated for 3 min and those for corticosteroids 60 min. For each line the linear regression equation and correlation coefficient are indicated. $N = 4$.

to angiotensin and ACTH. The prompt increase in cyclic AMP levels in response to angiotensin and ACTH was followed by an increase in steroidogenesis (Fig. 1, lower panel). Both ACTH and angiotensin, in the concentrations used in the cyclic AMP studies, were capable of inducing maximal rates of steroid accumulation which were established within 3 min and maintained at a constant rate for at least 12 min. Between 15 and 30 min the rates declined markedly and between 30 and 60 min were less than 10% of the maximal. Although in this particular experiment the steroidogenic response to angiotensin appeared to be slightly delayed as compared with the response to ACTH, this was not a consistent finding in other experiments.

Dose-response curve for angiotensin and ACTH on cyclic AMP accumulation. ACTH was clearly more effective than angiotensin in increasing cyclic AMP levels in cells incubated for 3 min (Fig. 2, upper panel). Half maximal levels of cyclic AMP were achieved with 4×10^{-9} M ACTH and 10^{-8} M angiotensin.

Dose-response curve for angiotensin and ACTH on corticosteroid accumulation. The steroidogenic responses of isolated cells incubated for 60 min in the presence of graded doses of angiotensin and ACTH are depicted in Fig. 2 (lower panel). ACTH was more potent in stimulating steroidogenesis at lower concentrations than angiotensin, but the slopes and maxima of the dose response curves generated by these two hormones were similar. Half maximal levels of fluorogenic corticosteroids were observed with 4×10^{-10} M ACTH and with 10^{-8} M angiotensin. When fractionated by thin-layer chromatography, 60–80% of the fluorogenic corticosteroids produced in response to either hormone were identified as cortisol, the remainder being corticosterone. The ratio of cortisol to corticosterone was the same for all doses of angiotensin and ACTH that were studied.

Correlation between corticosteroid and cyclic AMP levels. A close correlation between corticosteroids formed within 60 min and cyclic AMP accumulation during 3 min of incubation was observed in response to angiotensin stimulation (Fig. 3). A similar correlation between cyclic AMP and steroidogenesis was observed when ACTH was used as the stimulator. The data in Fig. 3 are derived from experiments shown in Fig. 2 but are depicted in this fashion in order to demonstrate that, regardless of which hormonal stimulator was used, there was virtual identity between the curves relating the cyclic AMP concentrations to steroid concentrations. These results are consistent with the view that the steroidogenic action of angiotensin, like that of ACTH, is mediated by cyclic AMP. After the plateau in the steroidogenic dose-response curve had been attained, higher doses of ACTH continued to increase cyclic AMP without further stimulation of steroidogenesis. This dissociation of effects was not observed with angiotensin.

Effect of repeated doses of angiotensin on cyclic AMP levels. To examine the possibility that the transience of the increase in cyclic AMP after angiotensin stimulation could be accounted for by rapid inactivation of the hormone, a second dose of angiotensin was added to the cells. When the second dose of angiotensin was added 6 min after the first dose, there was no increase in cyclic AMP during the ensuing 3 min (Fig. 4*C*). Addition of the second dose of angiotensin 12 min after the first dose had virtually no effect on cyclic AMP during the ensuing 6 min (Fig. 4 *D*). When the first dose of angiotensin was added 6 min after the incubation was begun, the cyclic AMP response was unaltered (Fig. 4 *B*), indicating that the delay in the response to a second dose of angiotensin was not due to damage of the cells during incubation.

Interaction of angiotensin and ACTH as stimulators of cyclic AMP accumulation. It was of interest to study the interaction of ACTH and angiotensin with respect to their effects on adrenal cell cyclic AMP. As illustrated in Fig. 5, the combination of hormones resulted in a cyclic AMP increase greater than the sum of the effects of each hormone alone ($P < 0.001$). These results indicate that at the time of their peak effects angiotensin and ACTH act synergistically in stimulating cyclic AMP formation in fasciculata cells. This is evidence that ACTH and angiotensin affect adrenal cell cyclic AMP through different mechanisms.

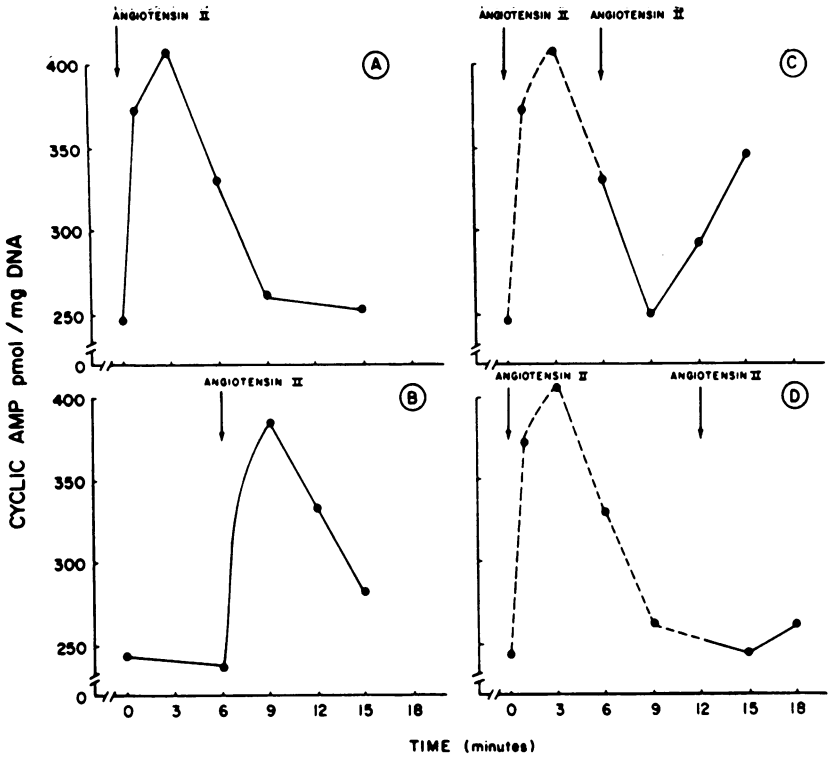

FIGURE 4 Effects of repeated doses of angiotensin on the time-course of cyclic AMP in cells plus medium. The arrows indicate the time at which 1 nmol of angiotensin (final concentration 10^{-6} M) was added to the cells. The incubation was carried out for the time intervals indicated. Each point represents the mean of two determinations.

Lack of effect of angiotensin on phosphodiesterase activity from fasciculata cells. With the observation of synergism between ACTH and angiotensin in elevating cyclic AMP, the question arose as to whether angiotensin inhibits phosphodiesterase activity. We, therefore, compared the effects of angiotensin with those of a known phosphodiesterase inhibitor (MIX) on the phosphodiesterase activity in 2,000 × *g* particles and 2,000 × *g* supernate. Whereas the xanthine derivative strongly inhibited the phosphodiesterase activity, angiotensin was without effect at a concentration that produced a maximal increase in cyclic AMP levels in intact cells.

Lack of effect of angiotensin on adenylate cyclase activity in washed particles. In order to examine whether angiotensin would activate adenylate cyclase, washed particles from fasciculata cells were incubated in the presence of angiotensin, ACTH, and fluoride. The rate of formation of cyclic AMP was linear for 12 min and was proportional to the amount of tissue used in the incubation. Both ACTH and fluoride increased cyclic AMP formation whereas angiotensin was without effect on adenylate cyclase activity under these conditions.

Interaction of angiotensin and ACTH as stimulators of steroidogenesis. Since both angiotensin and ACTH stimulate the synthesis of cortisol, and since they act synergistically to raise cyclic AMP levels, we examined their interaction as stimulators of steroid accumulation. As illustrated in Fig. 6, the combination of doses of angiotensin and ACTH which were maximally effective when the two agents were used separately stimulated no more steroidogenesis than was observed with ACTH alone. These data favor the idea that angiotensin and ACTH stimulate steroidogenesis in the same cell type. Otherwise an additive effect on steroid accumulation should have occurred. The combination of smaller doses of angiotensin and ACTH did result in addition of the effects observed when the two hormones were used separately (Fig. 6, lower panel).

DISCUSSION

The present study shows for the first time that angiotensin II increases cyclic AMP in bovine fasciculata cells while stimulating steroidogenesis (Figs. 1 and 2). Thus far, the stimulation of cortisol synthesis by angiotensin has been reported in only two species (6–9). It seems likely that the ability to synthesize cortisol in response to angiotensin is species specific since the human adrenal does not produce cortisol in response to angiotensin infusion (25, 26). However, the apparent failure

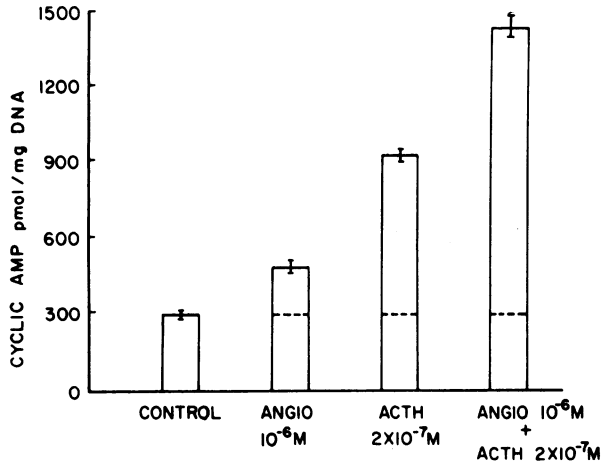

FIGURE 5 Combined action of angiotensin and ACTH on cyclic AMP accumulation in cells plus medium incubated for 3 min. $N = 8$.

of angiotensin to stimulate cortisol production in the human may be related to doses that can be safely administered to humans compared to other species.

The steroidogenic responses to angiotensin and ACTH were similar and probably occurred in the same cell type since the combination of maximal doses of both hormones did not result in an additive production of cortisol (Fig. 6). The rate of accumulation of steroids was constant for 15 min and declined thereafter (Fig. 1). The ratio of cortisol to corticosterone remained constant with increasing stimulation. The steroid response to either hormone correlated well with the increase in cyclic AMP until maximum steroidogenesis was achieved (Fig. 3). The fact that the increase in cyclic AMP occurred before the increase in steroidogenesis and the fact that the degree of increase in cyclic AMP was proportional to the increase in steroidogenesis are consistent with the view that cyclic AMP is the mediator of the steroidogenic action of angiotensin in bovine fasciculata cells. It would not be justifiable to extrapolate this conclusion to other species or other zones of the adrenal cortex.

Although in certain respects the effect of angiotensin on cyclic AMP is similar to that of ACTH, there are major differences in the effects of these hormones on adrenal cyclic AMP. Angiotensin induces a rapid increase in cyclic AMP (Fig. 1) which is small and transient when compared to that of ACTH. The disparity between the maximum cyclic AMP response to ACTH and angiotensin has possible precedents in the fact that the rat liver shows a greater maximal response to glucagon than to epinephrine (27) and the fact that rat kidney and bone show greater maximal cyclic AMP responses to parathyroid hormone than to calcitonin (28). The possibility that inactivation of angiotensin was responsible for the rapid decline of cyclic AMP was excluded when a second dose of angiotensin was added (Fig. 4) and found to be ineffective during the ensuing 3 min. This refractoriness of the cyclic AMP response to the continued presence of a hormone is reminiscent of what has been observed with other hormones in other tissues, suggesting that factors other than degradation of the stimulating agent might be responsible for the decline of cyclic AMP. Epinephrine in the dog heart (29), in fat cells (30, 31), and in the perfused rat liver (27) has been shown to produce only transient increases in cyclic AMP levels even in the presence of continuous stimulation. Although no unified explanation for the rapid decline of cyclic AMP in these different target organs is available, evidence for the formation of an inhibitor of adenylate cyclase has been presented in the case of fat cells (32).

The observation that angiotensin and ACTH act synergistically in increasing cyclic AMP (Fig. 5) cannot at present be explained by inhibition of phosphodiesterase activity by angiotensin nor by its activation of adenylate cyclase. If ACTH and angiotensin stimulated two sepa-

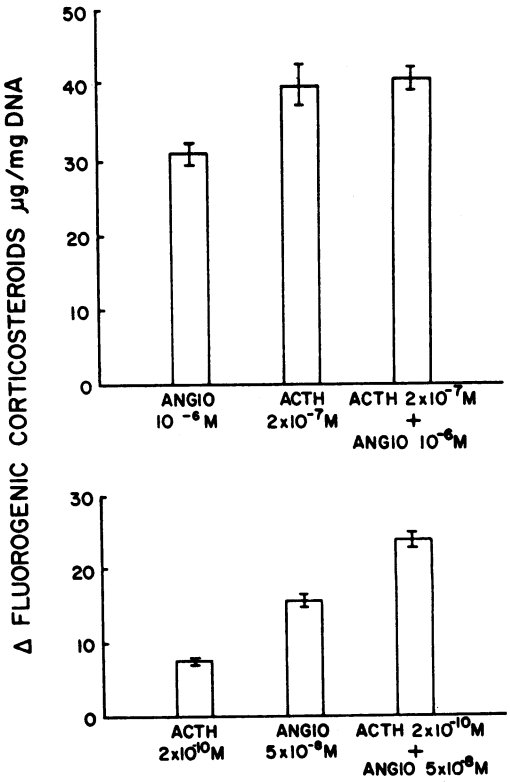

FIGURE 6 Effects of the combined action of angiotensin and ACTH on the net production of fluorogenic corticosteroids by fasciculata cells incubated for 60 min. The upper panel shows the effects of maximal doses ($N = 4$) and the lower panel the effects of submaximal doses ($N = 3$).

rate adenylate cyclase systems, this should only result in an additive effect on cyclic AMP and not in synergism. In fact, we were unable to demonstrate any activation of adenylate cyclase by angiotensin, although the possibility cannot be completely excluded since the in vitro conditions used may not have been optimal for such an effect.

In conclusion, our results indicate that angiotensin increases cyclic AMP while stimulating cortisol synthesis in bovine fasciculata cells. The close correlation between cyclic AMP and corticosteroid accumulation in response to angiotensin is consistent with the idea that angiotensin-induced steroidogenesis in fasciculata cells is mediated by cyclic AMP. However, the synergistic interaction of ACTH and angiotensin in elevating cyclic AMP levels suggests that the two hormones differ in the mechanisms through which they affect cyclic AMP metabolism.

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