The Role of Cyclic AMP in Aldosterone Production by Isolated Zona Glomerulosa Cells*

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The role of cyclic AMP in the regulation of aldosterone production by adrenocorticotropic hormone (ACTH), angiotensin II (A II), potassium, and serotonin was examined in collagenase-dispersed adrenal glomerulosa cells. The ability of 8-bromo cyclic AMP and choleragen to stimulate maximum aldosterone production indicated that cyclic AMP could act as second messenger for certain of the aldosterone-stimulating factors. The actions of ACTH and choleragen on aldosterone and cyclic AMP production were correlated in dog and rat cells, and a similar relation was seen during stimulation of rat cells by serotonin. In contrast, A II and potassium did not cause changes in cyclic AMP formation, whereas A II and potassium regulate aldosterone production. Intracellular and receptor-bound cyclic AMP were increased 3-fold by $10^{-7}$ M ACTH but not by A II. Addition of a phosphodiesterase inhibitor increased the magnitude of the cyclic AMP response to ACTH but did not change the lack of stimulation by A II or potassium. In dog cells, the effects of A II and potassium on aldosterone production were partially additive to those of ACTH, choleragen, and 8-bromo cyclic AMP. In contrast, no additivity was observed between A II and potassium, or between combinations of the cyclic AMP-dependent stimuli. These results indicate that the actions of ACTH on aldosterone secretion are mediated by cyclic AMP formation, whereas A II and potassium stimulate aldosterone production through an independent mechanism. The lack of additivity between steroid responses to A II and potassium suggests that these factors could share a common mode of action on steroidogenesis in zona glomerulosa cells.

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The abbreviations used are: ACTH, adrenocorticotropic hormone; albumin, bovine serum albumin.

MATERIALS AND METHODS

Synthetic [Asp', Ile']angiotensin II was obtained from Beckman Instruments Bioproducts Division, Palo Alto, CA. Porcine ACTH, serotonin, trypsin inhibitor, DNAse, and theophylline were obtained from Sigma, KC1 from Baker, medium 199 from Biochemical Associates, M3 choleragen from Schwarz/Mann, bovine serum albumin from Reheis Chemical, collagenase type II from Worthington, I-methyl-3-isobutylxanthine from Aldrich Chemical Co., and 8-bromo cyclic AMP from ICN Life Sciences.

Preparation of Isolated Glomerulosa Cells—To prepare isolated rat glomerulosa cells, animals were killed by carbon dioxide and the adrenal glands were removed and dissected free of fat. The capsular layer, consisting predominantly of zona glomerulosa cells, was separated from the underlying tissue by manual compression of each gland to extrude the fasciculata-reticularis zones and medulla from the capsule-glomerulosa tissue. The capsules were then finely minced with scissors and washed extensively with potassium-free medium 199. The washed tissue fragments were resuspended in 10 ml of medium 199 containing bovine serum albumin (2 mg/ml), collagenase (2 mg/ml), and 3.5 mM potassium, then gassed with 95% O2/5% CO2 and incubated with shaking at 37°C for 30 min. After this time, the tissue fragments were dispersed by aspiration through Tygon tubing into a plastic syringe as previously described (15, 16). The cell dispersion procedure was performed in medium 199 containing albumin (2 mg/ml), trypsin inhibitor (0.1 mg/ml), DNAse (5 µg/ml), and 3.5 mM potassium. The aspiration procedure was repeated until the cell suspension medium became clear, and the residual tissue fragments were subjected to a second period of collagenase digestion followed by dispersion as described above. The dispersed cells were pooled and centrifuged at 200 × g for 10 min at 4°C, then resuspended in the appropriate volume of incubation medium, which consisted of medium 199 containing 1.25 mM calcium, 5 mM potassium, and albumin 2 mg/ml. By this method, the usual yield from 100 rat adrenal glands was 8 to 10 million glomerulosa cells, which contained from 1 to 5% elevated endogenous cyclic AMP, as induced by serotonin (7), to stimulate aldosterone production by glomerulosa tissue and cells in vitro. In addition, the actions of ACTH upon steroidogenesis in zona fasciculata cells are believed to be mediated by cyclic AMP (8, 9), and there is evidence to suggest that the aldosterone response to ACTH in glomerulosa cells is similarly dependent on cyclic AMP (7, 10).

However, the mechanisms by which angiotensin II and potassium regulate aldosterone production are not clear, and there is conflicting evidence about the role of cyclic AMP in the action of these stimulating factors. Thus in some reports an action of angiotensin II on cyclic AMP formation has been observed (7, 11-13), whereas in others there has been no apparent effect of the octapeptide on cyclic AMP during stimulation of aldosterone production in vitro (10, 14). In order to clarify this aspect of the mechanism of action of angiotensin II, we have analyzed the effects of several agents known to stimulate steroidogenesis—angiotensin II, ACTH, potassium, serotonin, and choleragen upon the simultaneous production of aldosterone and cyclic AMP in isolated zona glomerulosa cells.
fasciculata cells on examination by light microscopy. For certain experiments, the adrenal capsular cells were filtered through 1-cm columns of Sephadex G-15 to remove fasciculata cells (17). Such glomerulosa cell preparations gave lower cyclic AMP and aldosterone responses than the original cell dispersions, but were employed for evaluation of ACTH effects on steroid and cyclic AMP production in the absence of fasciculata cells.

The preparation of isolated glomerulosa cells from dog adrenal glands was performed on tissues obtained from animals immediately after death from an overdose of sodium pentothal. Slices of the capsular tissue containing glomerulosa cells were minced, digested with collagenase, and dispersed into isolated cells as described above for rat capsular tissue (15, 16).

Incubation of Isolated Glomerulosa Cells—One-milliliter aliquots of incubation medium containing about 150,000 cells were incubated in polyethylene vials at 37°C under 95% O2/5% CO2 with shaking at 100 cycles/min. When cyclic AMP determinations were to be made, the incubation vials also contained 0.2 mM 1-methyl-3-isobutylxanthine (since preliminary experiments revealed that cyclic AMP production in basal and stimulated glomerulosa cells was considerably enhanced in the presence of phosphodiesterase inhibitors), and incubations were performed for a period of 30 min. When aldosterone determinations were to be performed, cells were incubated for 2 h in the absence of 1-methyl-3-isobutylxanthine, because phosphodiesterase inhibitors were found to inhibit the conversion of precursor steroids to aldosterone in vitro. It is not possible to correlate 1-methyl-3-isobutylxanthine-induced increases in cyclic AMP and steroid responses in adrenal glomerulosa cells, because of the direct effect of 1 methyl 3 isobutylxanthine in inhibiting the steroid biosynthetic pathway. All experiments were performed with duplicate incubation vials at each hormone concentration, and the cyclic AMP or aldosterone content, or both, of each vial was measured by radioimmunoassay of three aliquots of the incubation medium.

Following incubation, vials for cyclic AMP assay were transferred to an ice bath, and 99 ml of medium was immediately placed in glass tubes containing an equal volume of 2 ml methylenephine. The tubes were then placed in a boiling water bath for 10 min and centrifuged at 1500 x g for 10 min. For aldosterone determinations, media were decanted from incubation vials into glass tubes and centrifuged immediately at 1500 x g for 10 min. The maximal aldosterone and cyclic AMP responses and the concentration of the stimulators producing a half-maximal response (ED50) were determined by computer analysis using a four-parameter logistic function (18).

In Vivo Administration of Aldosterone Regulators—In addition to the in vitro experiments with isolated glomerulosa cells as described above, a series of in vivo studies was performed to evaluate the effects of angiotensin II, potassium, and ACTH upon adrenal steroidogenesis and cyclic AMP production in the zona glomerulosa. For this purpose, appropriate doses of angiotensin II, ACTH, and potassium were administered intravenously to 1-day hypophysectomized rats over a period of 30 min. The use of hypophysectomized animals in these experiments was chosen to avoid the possible complication of stress-induced ACTH stimulation during the procedure. For administration of the regulators, rats were anesthetized with sodium pentobarbitol and infused intravenously over a period of 30 min with the following agents: angiotensin II, 100 ng/min; KCl, 0.05 meq/min; ACTH, 10 ng/min. After administration of these doses of the individual stimulators, adrenal glands were removed, dissected free of fat, and expressed manually to separate the capsular zone from the underlying adrenal tissue. The pairs of adrenal capsules from individual experimental animals were minced and frozen rapidly on dry ice, and were later sonicated for 20 s prior to estimation of steroid and cyclic AMP content. For these experiments, we estimated the content of the precursor corticosterone in the adrenal capsules, rather than aldosterone. This was done because the heating step employed to preserve cyclic AMP in the tissue homogenate was found to cause degradation of aldosterone, whereas the effect of heating upon corticosterone was negligible.

Determination of Cyclic AMP and Aldosterone—Measurement of cyclic AMP was performed by a modification (19) of the radioimmunoassay method of Steiner et al. (20), with the addition of the acetylation step described by Harper and Brooker (21). The aldosterone content on incubation media was determined by direct radioimmunoassay of appropriate aliquots of the medium as previously described (15). Protein determinations were performed by the method of Lowry et al. (22), employing bovine serum albumin as standard.

RESULTS

Time Course of Cyclic AMP Production—In initial experiments, the rate of cyclic AMP production was examined during incubation of isolated glomerulosa cells with concentrations of the three regulators known to produce maximum stimulation of aldosterone production, i.e. 10^-9 M ACTH, 10^-9 M angiotensin II, and 15 mM potassium. As illustrated in Fig. 1, 10^-9 M ACTH caused a rapid increase in cyclic AMP production that became evident within 2 min and reached a plateau at 30 min. However, no effect of angiotensin II or increased potassium concentration was observed, and the cyclic AMP content in vials incubated with these stimuli remained at the basal level observed in control cells. The concentrations of ACTH, angiotensin II, and potassium employed in this experiment stimulated aldosterone production in a linear manner for up to 2 h of incubation. On the basis of these observations, in subsequent experiments the incubation was terminated at 30 min for cyclic AMP determinations, and at 120 min for measurement of aldosterone production.

Effects of ACTH, Serotonin, and Choleragen on Cyclic AMP and Aldosterone Production in Isolated Cells—The effects of ACTH on cyclic AMP and aldosterone production in dispersed rat glomerulosa cells are shown in Fig. 2, which illustrates the pooled dose-response curves obtained in six separate experiments. Stimulation of aldosterone production was evident at 10^-9 M ACTH and reached a maximum at 10^-8 M, with an ED50 of 4.5 X 10^-10 M ACTH. In the same experiments, stimulation of cyclic AMP production became apparent at 10^-10 M ACTH and reached a maximum at 10^-7 M, with ED50 of 6 X 10^-9 M ACTH. In one experiment, an increase in cyclic AMP was detected at 10^-11 M ACTH, at the same concentration that produced the first elevation of aldosterone production. However, there was in general a dissociation

![Fig. 1](left). Time course of cyclic AMP production in rat adrenal glomerulosa cells, in response to angiotensin II, ACTH, and potassium. Each data point represents the mean and S.E. of four determinations.

![Fig. 2](right). Dose-response curves for stimulation of aldosterone and cyclic AMP by ACTH in rat adrenal glomerulosa cells. Each point represents the mean and S.E. of the pooled data from six experiments.
between the aldosterone and cyclic AMP dose-response curves, as indicated by the almost 10-fold difference in ED$_{50}$ of ACTH for the two responses. Despite this difference in the two response curves, slight to marked increases in cyclic AMP production were evident over the ACTH concentration range from $10^{-10}$ to $10^{-7}$ M, which evoked the full aldosterone response in isolated glomerulosa cells. The cyclic AMP and aldosterone responses of Sephadex-purified glomerulosa cells are shown in Table I, illustrating the 4- to 5-fold increases in cyclic AMP production that accompanied the stimulation of steroidogenesis by ACTH. In dog adrenal glomerulosa cells, a similar degree of correlation between cyclic AMP and aldosterone production was observed during stimulation by ACTH (data not shown).

Serotonin is known to be an effective stimulus for aldosterone production in rat glomerulosa cells (5) and has been previously shown to cause an increase in cyclic AMP production in purified glomerulosa cells (7). In our studies, serotonin was also found to stimulate cyclic AMP production with a time course similar to that of ACTH, i.e., reaching a plateau in the first 30 to 60 min of incubation. Also, the magnitude of the cyclic AMP response to serotonin was consistently much smaller than the response evoked by ACTH. The effects of increasing concentrations of serotonin upon cyclic AMP and aldosterone production in dispersed rat glomerulosa cells are shown in Fig. 3, which illustrates that a marked increase in steroidogenesis was stimulated by serotonin over the concentration range from $10^{-9}$ to $10^{-5}$ M. At the same time, a less marked but consistent and serial increase in cyclic AMP production was observed, over the same concentration range required to evoke the steroid response. These results, based upon the data from three separate experiments, indicate the presence of a close relationship between the cyclic AMP and aldosterone responses to serotonin in isolated glomerulosa cells.

The effects of choleragen upon cyclic AMP and aldosterone production in isolated glomerulosa cells are shown in Fig. 4, which illustrates the pooled data obtained in three separate experiments. The aldosterone response became evident at $10^{-9}$ M choleragen and reached a maximum at $10^{-8}$ M. Over the same concentration range, there was a serial increase in cyclic AMP production, which continued to rise at choleragen concentrations above those necessary to elicit the full steroidogenic response and reached a maximum at about $3 \times 10^{-7}$ M choleragen (30 µg/ml). It should be noted that the increases in cyclic AMP production evoked by both choleragen and serotonin were relatively small, representing only about a doubling of the basal cyclic nucleotide level observed in unstimulated cells. This change is in contrast with the much larger increase in cyclic AMP production evoked by ACTH. However, the apparent differences between ACTH and the other cyclic AMP-stimulating agents may result in part from the different course of action of these regulators. As shown in Fig. 1, the cyclic AMP response to ACTH reached its maximum within about 30 min, whereas choleragen was found to evoke a much slower increase in cyclic AMP production (Fig. 5). For this reason, when the effects of choleragen upon glomerulosa cell responses were measured after a 3-h incubation, a much closer correspondence between cyclic AMP formation and aldosterone production was observed (Fig. 6).

**Effects of Angiotensin II and Potassium on Cyclic AMP and Aldosterone Production in Isolated Cells**—The pooled aldosterone dose-response curve of six experiments in which isolated glomerulosa cells were incubated with increasing concentrations on angiotensin II is shown in Fig. 7. Because the responsiveness of the individual cell preparations varied between experiments, the standard error of the pooled response curves is relatively large. However, within each individual response curve, the standard error of the replicates was quite small ($\pm 4.3\%$). Stimulation of aldosterone production was consistently detectable at angiotensin II concentrations as low as $10^{-11}$ M and reached maximum at $10^{-9}$ M, with an ED$_{50}$ of $2 \times 10^{-10}$ M angiotensin II. However, in the same experiments, there was no change in the cyclic AMP production by cells incubated with concentrations of angiotensin II over the same dose range, up to $10^{-5}$ M in these experiments and up to $10^{-4}$ M in others for which the data are not shown. Additional experiments were performed in the presence of 2 mM 1 methyl-3-isobutylxanthine instead of the 0.2 mM concentration employed in the preceding experiments, but again there was no detectable action of angiotensin II upon cyclic AMP production by isolated cells (Fig. 8). A similar lack of effect upon cyclic AMP in the presence of stimulation of a full steroidogenic response was observed during incubation of isolated glomerulosa cells with the des-Asp$^1$-heptapeptide of angiotensin II and when cells were incubated in 3.5 mM potassium instead of the usual 5 mM concentration employed in these studies (data not shown). Variations in the calcium concentration of the incubation media were also examined,

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**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Aldo (pg/10$^6$ cells)</th>
<th>cAMP (pmol/10$^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>ACTH A II</td>
<td>Basal ACTH A II</td>
</tr>
<tr>
<td>1</td>
<td>0.56, 1.62, 1.15</td>
<td>0.31, 1.63, 0.24</td>
</tr>
<tr>
<td>2</td>
<td>0.91, 5.21, 2.05</td>
<td>0.66, 2.44, 0.52</td>
</tr>
</tbody>
</table>

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**Fig. 3 (left).** Dose-response curves for stimulation of aldosterone and cyclic AMP production by serotonin in rat adrenal glomerulosa cells. Each point represents the mean and S.E. of the pooled data from three experiments.

**Fig. 4 (right).** Dose-response curves for stimulation of aldosterone and cyclic AMP production by choleragen in rat adrenal glomerulosa cells. Each point represents the mean and S.E. of the pooled data from three experiments.
Cyclic AMP and Aldosterone Production

**FIG. 5.** Time course of aldosterone and cyclic AMP production in rat glomerulosa cells stimulated by ACTH, serotonin (5-hydroxytryptamine, 5HT), and choleragen (CT). Each point represents the mean and S.E. of six determinations performed on duplicate incubation vials.

**FIG. 7.** Dose-response curves for stimulation of aldosterone production, and the corresponding cyclic AMP levels, in glomerulosa cells incubated with angiotensin II. Each point represents the mean and S.E. of the pooled data from six experiments.

**FIG. 8.** Cyclic AMP production by glomerulosa cells incubated with increasing concentrations of angiotensin II or ACTH in the presence and absence of 2 mM 1-methyl-3-isobutyl xanthine.
Table II

<table>
<thead>
<tr>
<th>Angiotensin II concentration</th>
<th>Calcium concentration (mM)</th>
<th>CAMP (pmol/ml)</th>
<th>Aldo (ng/ml)</th>
<th>CAMP (pmol/ml)</th>
<th>Aldo (ng/ml)</th>
<th>CAMP (pmol/ml)</th>
<th>Aldo (ng/ml)</th>
<th>CAMP (pmol/ml)</th>
<th>Aldo (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.44</td>
<td>0.23</td>
<td>0.38</td>
<td>0.94</td>
<td>0.45</td>
<td>0.87</td>
<td>0.56</td>
<td>0.73</td>
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<tr>
<td>10^{-10} M</td>
<td>0.6</td>
<td>0.38</td>
<td>0.94</td>
<td>0.45</td>
<td>0.87</td>
<td>0.66</td>
<td>2.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-9} M</td>
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<td>0.23</td>
<td>2.64</td>
<td>0.65</td>
<td>2.53</td>
<td>0.66</td>
<td>2.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-7} M</td>
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<td>0.36</td>
<td>0.58</td>
<td>0.43</td>
<td>2.46</td>
<td>0.46</td>
<td>4.69</td>
<td>0.64</td>
<td>4.90</td>
</tr>
</tbody>
</table>

Fig. 9. Time course of cyclic AMP production in canine adrenal fasciculata cells stimulated with ACTH and angiotensin II. The curves at left show the mean of duplicate incubations. The bars at right represent the stimulation of cortisol production measured after incubation for 30 min.

but in no case could an effect of angiotensin II upon cyclic AMP production be demonstrated (Table II).

In other experiments, measurements of intracellular cyclic AMP and protein-bound cyclic AMP were performed, the latter to evaluate cyclic AMP production and binding to the regulatory subunit of protein kinase, as previously described for the Leydig cell (23). However, although 10^{-7} M ACTH was found to cause increases in extracellular cyclic AMP (10 to 20 times above basal), intracellular cyclic AMP, and receptor-bound cyclic AMP (2 to 3 times above basal), no effects of angiotensin II were detected upon any of these pools of cyclic AMP in cells incubated in the same experiments with supramaximal steroidogenic concentrations of angiotensin II.

In another approach, we examined the effects of angiotensin II upon the steroidogenesis and cyclic AMP production in the dog zona fasciculata, employing dispersed cells prepared by the same techniques utilized for isolation of zona glomerulosa cells. This comparison was performed because angiotensin II is known to stimulate cortisol production in the canine zona fasciculata, and the actions of ACTH upon steroidogenesis in the fasciculata zone are clearly dependent upon cyclic AMP production (8, 9). However, although 10^{-7} M angiotensin II produced a 50% increase in cortisol production in canine fasciculata cells at 30 min of incubation, there was again no effect upon the level of cyclic AMP production. In the same cells, 10^{-8} M ACTH produced an early and marked increase in cyclic AMP, with a similar steroid response to that evoked by angiotensin II (Fig. 9). Thus, under a variety of conditions as described above, it was possible not to observe an increase in cyclic AMP production during stimulation of steroidogenesis in isolated adrenal cells by angiotensin II. In contrast, under the same experimental conditions, increases in cyclic AMP formation were always demonstrable during incubation with stimuli such as ACTH, serotonin, and choleragen.

The effects of extracellular potassium concentration upon aldosterone production and cyclic AMP formation are shown in Fig. 10, which compares the actions of 5 and 15 mM K+ upon these responses. It is evident that the marked increase in aldosterone production elicited by incubation of isolated cells with 15 mM potassium is not accompanied by a change in cyclic AMP production by the same cells. As with angiotensin II, it has not been possible in these experiments to demonstrate any effect of altered medium potassium concentrations upon cyclic AMP formation and production, despite simultaneous stimulation of aldosterone production by the isolated cells. Similarly, no change in cyclic AMP formation was observed in the presence of 2 mM 1-methyl-3-isobutylxanthine when glomerulosa cells were incubated with high potassium concentrations (data not shown).

Additivity between Stimuli of Aldosterone Production—To examine further the possibility that the actions of angiotensin II and potassium are mediated by mechanisms which differ from that of ACTH, isolated canine glomerulosa cells were incubated with combinations of the individual regulators at concentrations known to produce a maximum effect upon steroidogenesis. It might be expected that the existence of such a difference in mechanism would result in additivity of the steroidogenic response when cells were incubated with...
stimuli acting through the separate mechanisms. The results of three such experiments, shown in Fig. 11, illustrate that partial additive effects were indeed detectable when angiotensin II or potassium was added to cells simultaneously incubated with ACTH. In contrast, no additivity was demonstrable between angiotensin II and potassium, suggesting that these stimuli may act by a common mechanism and thus do not cause an increased response when present at maximal stimulating concentrations.

In a further series of experiments, additive effects were sought between additional cyclic AMP-dependent stimuli (choleragen and 8-bromo cyclic AMP) and the steroidogenic actions of angiotensin II and potassium. As shown in Fig. 12, in which the steroid response to $10^{-5}$ M 8-bromo cyclic AMP was taken as 100%, no additivity was observed with combinations of ACTH and the cyclic AMP-dependent stimulants, whereas a small but significant degree of additivity was evident when angiotensin II or potassium was combined with either of the cyclic AMP-dependent stimuli.

**In Vivo Effects of Aldosterone Regulators**—In these experiments, shown in Fig. 13, no effect of angiotensin II or potassium upon zona glomerulosa cyclic AMP levels was detectable, whereas a significant increase in cyclic AMP content was evident after infusion of ACTH. As noted above during the in vitro experiments with dispersed glomerulosa cells, each stimulator produced a marked increase in steroidogenesis, as shown in this case by the increased concentrations of corticosterone in adrenal capsular tissue. In the same experiments, ACTH was found to cause a more marked increase in cyclic AMP content in the fasciculata-reticularis cells than in the capsular layer, and the administration of either angiotensin II or potassium caused no change in the cyclic AMP content of the fasciculata-reticularis layers (data not shown). Thus the results of these in vivo experiments were consistent with the observations made in vitro upon dispersed glomerulosa cells. In both cases, ACTH had a marked and significant effect upon both cyclic AMP and steroidogenesis, but no changes in cyclic AMP concentration was elicited by potassium and angiotensin II despite the marked steroidogenic response to each of these stimuli.

**DISCUSSION**

These studies were performed to provide more detailed information about the role of cyclic AMP in aldosterone production by comparing the effects of the three classical regulators of aldosterone production (angiotensin II, ACTH, and potassium) upon cyclic AMP levels in zona glomerulosa cells exposed to these stimuli in vitro and in vivo. The results of previous studies performed on this topic have been inconsistent, and a degree of uncertainty has remained about the involvement of cyclic AMP in the steroidogenic response of the zona glomerulosa to angiotensin II and potassium. For this reason, we have evaluated the actions of these regulators in vitro in cells derived from the zona glomerulosa of the canine and rat adrenal glands. When these studies were performed under identical experimental conditions for each regulator, it was apparent that the action of ACTH upon aldosterone production in vitro and in vivo was accompanied by readily detectable changes in cyclic AMP, consistent with the view that the mechanism of action of ACTH upon adrenal steroidogenesis is mediated by the adenylate cyclase-protein kinase system with cyclic AMP as the second messenger (8, 9, 24). The cyclic AMP response to ACTH was also observed in Sephadex-purified rat glomerulosa cells, indicating that the small proportion of fasciculata cells present in capsular cell dispersions was not responsible for the cyclic AMP increases during ACTH stimulation. Also, ACTH consistently stimulated cyclic AMP production in dog glomerulosa cells, in which the absence of fasciculata cell contamination was indicated by the failure of ACTH to elicit cortisol production during stimulation of aldosterone synthesis.

The actions of other stimuli of aldosterone production that are believed to operate through cyclic AMP, i.e., choleragen and serotonin, were also found to be accompanied by a progressive rise in cyclic AMP formation over the concentration range that elicited a steroidogenic response in isolated glomerulosa cells. The serotonin-stimulated cyclic AMP response provides further evidence for the ability of glomerulosa cells to respond to certain stimuli with a rise in cyclic nucleotide production, since serotonin has no effect on cyclic AMP and steroid production in adrenal fasciculata cells (4). Thus changes in cyclic AMP formation were readily detectable in glomerulosa cells stimulated by factors that are known to regulate steroidogenesis predominantly through an effect upon adenylate cyclase.

In contrast to these findings, there was no evidence for an effect of either angiotensin II or potassium upon cyclic AMP formation in glomerulosa cells stimulated both in vitro and in vivo by concentrations of these agents that caused a marked increase in aldosterone production. The absence of detectable changes in cyclic AMP formation during stimulation of aldosterone production by angiotensin II was consistently noted under conditions which revealed easily detectable effects of
the regulators acting through cyclic AMP. This disparity is consistent with the view that both angiotensin II and potassium exert effects upon aldosterone production through a mechanism that is independent of cyclic AMP production and the classical adenylyl cyclase protein kinase pathway. The nature of this alternative mechanism for activation of steroidogenesis has yet to be clarified, but the importance of calcium in this process has been emphasized by recent studies which demonstrated marked calcium dependence of the actions of angiotensin II and potassium during stimulation of aldosterone production in isolated glomerulosa cells (25).

Further evidence for the existence of the two pathways was provided by the additivity experiments performed in vitro, in which the effects of supramaximal steroidogenic concentrations of angiotensin II and potassium were found to be additive with the effect of ACTH, choleragen, and 8-bromo cyclic AMP, whereas no additivity was observed when cells were stimulated by both angiotensin II and potassium. These experiments were performed with canine zona glomerulosa cells to avoid the complication of endogenous precursor production during incubation with the cyclic AMP-dependent stimuli. If such experiments were performed with rat glomerulosa cells, the presence of a small degree of contamination with fasciculata cells could contribute significant quantities of corticosterone during incubation with ACTH and thus accentuate the apparent effects of the stimuli. However, dog fasciculata cells produce cortisol as the predominant glucocorticoid, and such contamination for the discrepancy that exists between published reports concerning the relationship of cyclic AMP to angiotensin II-induced aldosterone production in the zona glomerulosa is independent of cyclic AMP formation. However, it is clear that a cyclic AMP-mediated pathway exists in these cells and can be stimulated by the appropriate regulators, of which the physiologically relevant agent is ACTH. In regard to the action of potassium, we observed no consistent changes in cyclic AMP production during acute exposure of cells to high potassium levels. Although small changes in cyclic AMP have been described in earlier reports (10, 12), our studies have shown either insignificant changes or no effect as demonstrated in the present report. Also, the previously observed changes in cyclic AMP have not correlated with the effects of physiological changes in potassium upon aldosterone production. For these reasons, it is most unlikely that the action of potassium on aldosterone secretion is mediated through a change in cyclic AMP production.

Since the proximate regulation of aldosterone production by the adrenal gland under normal physiological conditions is predominately exerted through the renin-angiotensin system, especially during states of sodium deficiency and excess, it is particularly important to clarify the mechanism by which angiotensin II activates aldosterone production in the zona glomerulosa. While the present studies have not provided an explanation for this action of angiotensin II, it is clear that increased cyclic AMP production does not have an obligatory role in this process. Alternative possibilities include the redistribution of intracellular cyclic AMP pools, or more likely, the existence of a calcium-dependent mechanism that does not involve the cyclic AMP system (25). Whatever the nature of this activation pathway, it appears likely to be shared by angiotensin II and potassium, since the lack of additivity between the steroid responses to these agents suggests that they possess a common mechanism of action upon steroidogenesis in the zona glomerulosa.

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