Adenosine 3':5'-Cyclic Monophosphate Production and Steroidogenesis by Isolated Rat Adrenal Glomerulosa Cells

EFFECTS OF ANGIOTENSIN II AND [SAR¹,ALA⁸]ANGIOTENSIN II

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Angiotensin II effects on cyclic AMP production and steroid output were studied in a sensitive preparation of isolated rat adrenal glomerulosa cells. With increasing concentrations of angiotensin II logarithmic dose-response curves for aldosterone and cyclic AMP production were similar. The minimum effective dose (0.2 nM) for stimulation of aldosterone production also significantly ($P < 0.001$) increased cyclic AMP output. For both aldosterone and cyclic AMP production, the peptide hormone concentration eliciting maximal response (0.2 μ M) and the ED₅₀ (median effective dose) values (1 nM) were the same; this is consistent with cyclic AMP acting as an intracellular mediator for angiotensin II-stimulated aldosterone production by glomerulosa cells. The angiotensin II antagonist [Sar¹,Ala⁸]angiotensin II inhibited angiotensin II-stimulated corticosterone and aldosterone production in these cells. An equimolar concentration of antagonist halved the response to 20 nM-angiotensin II, and complete inhibition was observed with 0.2 μ M-antagonist. In contrast, [Sar¹,Ala⁸]angiotensin II had no effect on maximally stimulated steroidogenesis induced by serotonin and a raised extracellular K⁺ concentration. Increasing concentrations of [Sar¹,Ala⁸]angiotensin II alone decreased corticosterone and aldosterone outputs significantly ($P < 0.05$) at concentrations of 20 nM and 2 nM of antagonist respectively. A significant ($P < 0.001$) decrease in cyclic AMP production occurred with 2 μ M antagonist and this was comparable with the decrease in aldosterone production. It is concluded that [Sar¹,Ala⁸]angiotensin II can independently affect glomerulosa-cell steroidogenesis, possibly by modulating adenylate cyclase activity.

The considerable evidence supporting the concept that the adrenal corticosteroidogenic action of corticotropin (ACTH) is mediated by changes in intracellular cyclic AMP concentrations has been reviewed (Robison *et al.*, 1971; Schulster *et al.*, 1976; Halkerston, 1976). However, the mechanism whereby angiotensin II stimulates adrenal aldosterone production is less well established, particularly with regard to the role of cyclic nucleotides. Peytremann *et al.* (1973) showed that in isolated fasciculata cells angiotensin II elicits an increase in cyclic AMP output that precedes a stimulation of corticoid synthesis. Aldosterone is produced only by the zona glomerulosa of the adrenal cortex, and the renin-angiotensin system is one (and in some species perhaps the principal) mechanism controlling aldosterone biosynthesis *in vivo*. Exogenous cyclic AMP has been shown to stimulate aldosterone production in bovine adrenal slices, rat adrenal quarters and both unpurified and purified isolated

rat adrenal glomerulosa cells (Kaplan & Cook, 1965; Müller, 1970; Haning *et al.*, 1970; J. F. Tait *et al.*, 1974). However, concentrations of angiotensin II that maximally stimulated aldosterone production failed to increase the cyclic AMP content of either outer bovine adrenal slices or isolated cells prepared from such slices (Saruta *et al.*, 1972; Peytremann *et al.*, 1974). Cyclic AMP production by purified dispersed rat adrenal glomerulosa cells was increased by angiotensin II, but only at those concentrations (0.4 mM and 0.8 mM) that produced maximal and similar (2-fold) stimulation of corticosterone production (Albano *et al.*, 1974; S. A. S. Tait *et al.*, 1974).

We have described (Bing & Schulster, 1977) a sensitive isolated rat adrenal glomerulosa-cell system that, like a previously reported preparation of isolated dog glomerulosa cells (Fredlund *et al.*, 1975), responds with increased corticosterone and aldosterone production to angiotensin II concentrations (20 pM and 0.2 nM) within or just above normal plasma concentrations. We now report a comparison of aldosterone and cyclic AMP outputs by this rat glomerulosa-cell preparation over a range of

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angiotensin II concentrations. The effects of [Sar¹,Ala⁸]angiotensin II, a competitive inhibitor of the peptide hormone (Peach & Chiu, 1974; Williams *et al.*, 1974; Hollenberg *et al.*, 1976; Saltman *et al.*, 1976; Douglas *et al.*, 1976), on basal and stimulated steroidogenesis and on basal cyclic AMP output are examined. Some of this work has been presented in a preliminary form (Bing & Schulster, 1976). The findings are consistent with an intermediary role for cyclic AMP in the angiotensin II stimulation of aldosterone production by the adrenal glomerulosa.

Experimental

Materials

Angiotensin II (free acid; Asp¹,Val⁵; M.R.C. Research Standard A) was supplied by the W.H.O. International Laboratory for Biological Standards, Hampstead, London N.W.3, U.K. The analogue [Sar¹,Ala⁸]angiotensin II was given by Professor J. D. Swales. Other materials were obtained as follows. Bovine serum albumin (fraction V; Armour Pharmaceuticals, Hampden Park, Eastbourne, Sussex, U.K.); collagenase (CLS 138 units/mg from *Clostridium histolyticum*; Cambrian Chemicals, Croydon, London, U.K.); 5-hydroxytryptamine (serotonin; Sigma Chemical Co., Poole, Dorset, U.K.); cyclic AMP (Calbiochem, Bishops Cleeve, Herts., U.K.); Dowex AG 50W-X4 (200–400 mesh; BioRad Laboratories, Bromley, Kent, U.K.); [8-³H]Adenosine 3':5'-cyclic monophosphate (sp. radioactivity 27 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

The methodology for the preparation of isolated rat adrenal glomerulosa cells was as previously described (Bing & Schulster, 1977) and was based on methodology used in this laboratory for the preparation of isolated adrenal fasciculata cells (Richardson & Schulster, 1972). The buffer medium (KRBGA buffer) used throughout these studies was Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing glucose (0.2%, w/v), bovine serum albumin (0.5%, w/v) and modified to give a final K⁺ concentration of 3.6 mM. Incubations of glomerulosa cells in medium containing 3.6 mM-K⁺ have been shown by others (Tait *et al.*, 1972; Fredlund *et al.*, 1975) and confirmed by ourselves (Bing & Schulster, 1977) to give the lowest outputs of both corticosterone and aldosterone; such conditions were therefore considered to provide those for basal steroid production.

Normal adrenal capsular tissue obtained from freshly decapitated female Sprague-Dawley rats was subjected to digestion with collagenase (2 mg/ml)

and mechanical disruption by repeated aspiration into a glass pipette. After centrifugation (100g, 10 min, 37°C) and washing twice with fresh KRBGA buffer, cells were resuspended in KRBGA buffer to give the desired concentration (see Figure legends). The cell yield was 1×10^5 – 2×10^5 cells per adrenal, being composed predominantly of glomerulosa cells with less than 5% fasciculata-reticularis cell contamination. The physical characteristics of these cells and steroidogenic responses to various stimulators have been described (Bing & Schulster, 1977).

Cells were incubated in clear plastic tubes at 37°C in an atmosphere of O₂/CO₂ (19:1). The incubation volume was 1 ml [0.95 ml of cell suspension in KRBGA buffer and 0.05 ml of NaCl (0.9%, w/v) containing added reagents]. After incubation, either the aldosterone and corticosterone or the aldosterone and cyclic AMP content (cells plus supernatant) were measured in samples from all incubations in each experiment.

Aldosterone was measured, in triplicate, by a sensitive radiochemical immunoassay (Tait *et al.*, 1972). Cross-reactivity with corticosterone is less than 0.1% over the range 0.05–100 ng. The corticosterone content was determined fluorimetrically on a single sample from each incubation (Sayers *et al.*, 1971).

Cyclic AMP content was determined by a method established in the laboratory by using ion-exchange chromatography and an adrenal cyclic AMP-binding protein (Montague & Cook, 1971; Mackie *et al.*, 1972), which permit separation of cyclic AMP from other cyclic nucleotides. To each sample (0.9 ml of the incubate), 6% (w/v) trichloroacetic acid was added to precipitate proteins, and after centrifugation (1500g, 10 min, 4°C) the supernatant was chromatographed on a column (0.5 cm × 5 cm) of Dowex AG 50W-X4 (200–400 mesh). The eluate containing cyclic AMP was freeze-dried and redissolved in Tris/HCl buffer (50 mM, pH 7.4). The cyclic AMP in these extracts was measured in quadruplicate by saturation analysis by using a cyclic AMP-binding protein obtained from bovine adrenal-gland homogenates (Brown *et al.*, 1971). Dowex AG 50W-X4 was substituted for the Dowex AG 50W-X8 used previously to allow more rapid chromatography. Losses during these procedures were determined by the addition of a small amount of cyclic [³H]AMP to each sample and recoveries were about 70%.

Statistical analysis was by the Student's paired *t* test.

Results

The effects of increasing concentrations of angiotensin II on corticosterone and aldosterone production are shown in Fig. 1. [Asp¹,Val⁵]-

Fig. 1. Effect of angiotensin II on corticosterone (●) and aldosterone (○) production in isolated glomerulosa cells

Results are expressed as ng/h per adrenal and the values represent means $\pm$ S.E. for 6 experiments in duplicate, containing cells from half an adrenal gland. All stimulated outputs are significantly greater than basal amounts ($P < 0.05$), except for aldosterone at 20 pM-angiotensin II.

Angiotensin II (free acid; M.R.C. Research Standard A) was used throughout these studies because Bing & Schulster (1977) showed that it had no effect on corticosteroidogenesis in fasciculata-reticularis cells even at concentrations as high as 0.2 mM. The contribution of these contaminating cells to the steroid content of this preparation of glomerulosa cells is thus negligible. The production of both corticosterone and aldosterone is progressively stimulated with angiotensin II concentrations from 20 pM to 0.2 μ M. The variability in basal outputs of different cell preparations is considerable and not wholly dependent on the total number of cells present, as viability is difficult to assess. However, the pattern of response in different experiments was very similar (Kendall's coefficient of concordance $P < 0.01$). We and others have shown that the use of the ratio of stimulated to basal output allows comparison of results from experiments carried out on different occasions (J. F. Tait *et al.*, 1974; Bing & Schulster, 1977). Subsequent results are therefore shown in this form.

Table 1 gives the corticosterone and aldosterone productions as ratios in response to increasing concentrations of angiotensin II in greater detail than shown in Fig. 1. The response patterns for these two steroids were very similar except at the lowest concentration of hormone used. Corticosterone output was consistently and significantly ($P < 0.05$) increased at 20 pM-angiotensin II, whereas aldosterone production remained at basal concentrations. The

Table 1. Effects of angiotensin II on aldosterone and corticosterone production in isolated adrenal glomerulosa cells

Values represent means $\pm$ S.E. for 20 (10 experiments) 2h incubations of cells from half an adrenal gland. Basal outputs (mean $\pm$ S.E.) were 23.1 ± 2.7 ng of corticosterone/h per adrenal and 1.18 ± 0.21 ng of aldosterone/h per adrenal. * $P < 0.05$; significantly different from basal value.

Angiotensin II (M)	Steroid production (stimulated/basal)	
	Aldosterone	Corticosterone
0	1.00	1.00
2×10^{-11}	1.03 ± 0.05	$1.12 \pm 0.04^*$
2×10^{-10}	$1.31 \pm 0.06^*$	$1.28 \pm 0.04^*$
2×10^{-9}	$1.91 \pm 0.11^*$	$1.45 \pm 0.05^*$
2×10^{-8}	$2.15 \pm 0.10^*$	$1.63 \pm 0.04^*$
2×10^{-7}	$2.25 \pm 0.10^*$	$1.66 \pm 0.04^*$
2×10^{-6}	$1.94 \pm 0.15^*$	$1.58 \pm 0.05^*$

aldosterone response was significant at 0.2 nM-angiotensin II and production of both steroids was maximally stimulated by 0.2 μ M-peptide; and the ED₅₀ (concentration eliciting half-maximal response) was 0.36 nM- and 0.6 nM-angiotensin II for corticosterone and aldosterone respectively.

Logarithmic dose-response curves of cyclic AMP and aldosterone production after incubation with increasing concentrations of angiotensin II are shown in Fig. 2. The minimum concentration of hormone that increased aldosterone production (0.2 nM) also produced a significant ($P < 0.001$) increase in cyclic AMP output. The ED₅₀ for both cyclic AMP and aldosterone responses was 1 nM-angiotensin II, and maximal stimulation of both responses occurred at 0.2 μ M-angiotensin II.

[Sar¹,Ala⁸]Angiotensin II progressively inhibited the stimulation of steroidogenesis by 20 nM-angiotensin II (minimum concentration for maximal stimulation, see Table 1); 50% inhibition occurred with equimolar concentrations of antagonist and agonist, and complete inhibition was observed with 0.2 μ M-antagonist (Fig. 3). When increasing concentrations of [Sar¹,Ala⁸]angiotensin II were added alone to the glomerulosa cells, a progressive fall in corticosterone and aldosterone outputs occurred and this inhibition was significant ($P < 0.05$) at 20 nM and 2 nM concentrations of analogue respectively.

Cyclic AMP production was significantly ($P < 0.001$) decreased by 2 μ M-[Sar¹,Ala⁸]angiotensin II, and this decrease was comparable with the decrease in aldosterone output observed with this concentration of antagonist (Fig. 4).

Serotonin (1 μ M) and raised extracellular K⁺ concentration (6.0 mM) stimulate maximally corticosterone and aldosterone production in this glomeru-

Fig. 2. Cyclic AMP (●) and aldosterone (○) responses to angiotensin II

Each incubation tube contains cells equivalent to three adrenal glands. All stimulated values, expressed as ratios of stimulated/basal output and representing means $\pm$ s.e. of at least six (three experiments) 1 h incubations, are significantly greater than basal values ($P < 0.001$). The lowest concentration of angiotensin II that stimulates aldosterone (0.2 nM) also significantly ($P < 0.001$) increases cyclic AMP output. Basal values (means $\pm$ s.e.) were 4.06 ± 0.42 ng of aldosterone/h per adrenal and 0.73 ± 0.12 pmol of cyclic AMP/h per adrenal.

losa cell preparation (Bing & Schulster, 1977). $[\text{Sar}^1, \text{Ala}^8]$ Angiotensin II produced no change in the steroid response to these concentrations of serotonin and K^+ even at $2 \mu\text{M}$ concentration (Table 2). This applied to both the absolute amount of steroid produced and the ratio of stimulated to basal values.

Discussion

This isolated rat adrenal glomerulosa-cell preparation responds to physiological concentrations of angiotensin II with increased corticosterone and aldosterone production. Although a close agreement exists between the changes in the production rates of these two steroids with increasing concentrations of angiotensin II, there is an unexplained dissociation of the responses at the lowest concentration of hormone used.

The maximal stimulation of aldosterone of twice basal amounts contrasts with that of approximately eight times observed with acute angiotensin II infusions in conscious rats (Coleman *et al.*, 1974). Such discrepancies have been observed with other systems *in vitro*, and may be due to problems associated with the use of static incubation systems *in vitro* as opposed to dynamic perfusion systems *in vivo*. A dynamic superfusion system *in vitro* (Schulster & Jenner, 1975) may be one way to overcome this discrepancy.

A close correlation between changes in cyclic AMP and aldosterone production has been demonstrated with various concentrations of angiotensin II (Fig. 2).

Fig. 3. Effect of $[\text{Sar}^1, \text{Ala}^8]$ angiotensin II on basal (▲) and angiotensin II-stimulated (△) output of corticosterone (a) and aldosterone (b)

Values represent means $\pm$ s.e. of six (three experiments) 2 h incubations of cells from half an adrenal gland, expressed as a ratio of stimulated/basal output. Half-maximal inhibition of stimulated increases in both steroids was observed with equimolar concentrations of antagonist and agonist (20 nM). Antagonist alone decreases corticosterone and aldosterone production significantly ($P < 0.05$) at 20 nM and 2 nM respectively.

Fig. 4. Basal (a) and $2\mu\text{M}$ -[Sar¹,Ala⁸]angiotensin II-stimulated (b) values of cyclic AMP (open columns) and aldosterone (hatched columns) outputs

Means $\pm$ S.E. for nine (three experiments) 1 h incubations containing cells from the equivalent of three adrenal glands are shown. Values are ratios of stimulated/basal outputs. The decrease of both with the antagonist is significant ($P < 0.001$).

In particular, the minimum effective concentration of angiotensin II for stimulation of aldosterone also significantly increased cyclic AMP production; moreover, the ED_{50} and concentration eliciting maximal stimulation were the same for both cyclic AMP and aldosterone production. A further increase in cyclic AMP production did not occur with angiotensin II concentrations greater than those resulting in maximal steroidogenesis. This contrasts with the effect of corticotropin on isolated fasciculata cells, in which concentrations of corticotropin greater than those eliciting maximal steroidogenesis

were able to stimulate much greater cyclic AMP outputs (Mackie *et al.*, 1972; Beall & Sayers, 1972). No separation of angiotensin II-induced responses in aldosterone and cyclic AMP production was observed. These results are therefore entirely consistent with an intermediary role for cyclic AMP in the action of angiotensin II on aldosterone production by adrenal glomerulosa cells.

All non-purified preparations of glomerulosa cells are contaminated to some extent with fasciculata-reticularis cells, which can be stimulated to increase steroidogenesis by high concentrations of hormone (Peytremann *et al.*, 1973; S. A. S. Tait *et al.*, 1974). It is possible for corticosteroids secreted from fasciculata-reticularis cells to be taken up by the glomerulosa cells in such a dispersed mixed cell system and then be converted into aldosterone (Haning *et al.*, 1970; Williams *et al.*, 1972). Previous studies with insensitive mixed cell suspensions and high angiotensin II concentrations (i.e. in the μM range) are thus open to criticism. Responses of isolated glomerulosa cells, purified by unit-gravity sedimentation, have been examined (Albano *et al.*, 1974; S. A. S. Tait *et al.*, 1974), but only the effect of very high angiotensin II concentrations ($>100\mu\text{M}$) were reported; threshold sensitivities of these purified rat glomerulosa cells were not published. Other investigators have reported dispersed rat (Williams *et al.*, 1974) and dog (Fredlund *et al.*, 1975) glomerulosa-cell preparations responsive to normal plasma concentrations of angiotensin II (about 20–50 pM), although these studies did not report the degree of fasciculata-reticularis cell contamination. Our studies have utilized an isolated rat glomerulosa-cell suspension that responds to concentrations of angiotensin II free acid in the physiological range, and is contaminated with less than 5% fasciculata-reticularis cells (Bing & Schulster, 1977). Similarly prepared

Table 2. Effect of [Sar¹,Ala⁸]angiotensin II on basal and maximally stimulated steroidogenesis by serotonin and K⁺ Aldosterone (Aldo.) and corticosterone (Cort.) outputs expressed as ratios of stimulated to basal production are shown in response to serotonin ($1\mu\text{M}$) and K⁺ (6.0 mM) in the presence of [Sar¹,Ala⁸]angiotensin II. Values are means $\pm$ S.E. for 8 (4 experiments) 2 h incubations containing cells from half an adrenal gland. The basal steroid outputs were aldosterone 2.81 ± 0.25 ng/h per adrenal and corticosterone 60.6 ± 9.4 ng/h per adrenal. * indicates significant ($P < 0.05$) decrease from incubations without antagonist added.

		Steroid output (stimulated/basal)				
[Sar ^I ,Ala ⁸]Angiotensin II concentration (nM)		...	0	0.2	20	2000
Steroid measured						
Basal (3.6mM-K ⁺)	Aldo.		1.00	0.91 ± 0.11	0.67 ± 0.04*	0.63 ± 0.04*
	Cort.		1.00	1.00 ± 0.02	0.86 ± 0.04*	0.80 ± 0.05*
K ⁺ (6.0mM-K ⁺)	Aldo.		8.12 ± 0.36	7.85 ± 0.23	7.49 ± 0.51	7.60 ± 0.56
	Cort.		2.62 ± 0.09	2.47 ± 0.05	2.49 ± 0.03	2.32 ± 0.07
Serotonin (1 μM)	Aldo.		8.44 ± 0.33	9.13 ± 0.44	8.44 ± 0.25	8.78 ± 0.56
	Cort.		2.53 ± 0.14	2.53 ± 0.14	2.50 ± 0.15	2.45 ± 0.14

fasciculata-reticularis cells showed no corticosteroidogenic response to these, and higher concentrations of angiotensin II free acid (up to 0.2 mM). It may be the combined use of the angiotensin II free acid, together with this sensitive glomerulosa-cell preparation and short incubation times, that has now allowed the demonstration of a close correlation between changes in cyclic AMP and aldosterone production (Fig. 2).

[Sar¹,Ala⁸]Angiotensin II has been shown to inhibit competitively the action of angiotensin II on blood pressure and aldosterone production both *in vivo* and *in vitro* (Peach & Chiu, 1974; Williams *et al.*, 1974; Hollenberg *et al.*, 1976; Saltman *et al.*, 1976; Douglas *et al.*, 1976), and to displace angiotensin II from adrenal plasma-membrane preparations (Douglas *et al.*, 1976; Park *et al.*, 1973; Mimran *et al.*, 1974; Saltman *et al.*, 1975). The present studies confirm its inhibitory effect on angiotensin II stimulation of aldosterone. The stimulation in output of corticosterone and aldosterone, induced by angiotensin II, was inhibited by 50% when an antagonist/agonist ratio of 1:1 was used (Fig. 3). This is similar to previous observations with isolated rat glomerulosa cells (Williams *et al.*, 1974). However, an antagonist/agonist ratio of 50:1 was required for 50% inhibition of maximal angiotensin II-stimulated aldosterone production in isolated dog glomerulosa cells (Saltman *et al.*, 1976) and a 17:1 ratio for 50% inhibition of specific angiotensin II binding to dog adrenal plasma membranes (Douglas *et al.*, 1976). These differences in inhibitory potency of this analogue may be due to species differences.

The decrease of basal steroid output caused by [Sar¹,Ala⁸]angiotensin II in the absence of angiotensin II was surprising. A similar decrease in steroid production with this antagonist has been reported in isolated rat glomerulosa cells (Williams *et al.*, 1974), but was not seen with isolated dog glomerulosa cells (Saltman *et al.*, 1976). One possibility considered was that this effect was due to displacement of angiotensin II still bound to cells. However, this is unlikely, since the preparation of the cell suspension involves several washes with fresh buffer and we have observed in other experiments that one such wash is sufficient to remove all detectable [³H]-angiotensin II. Also a direct cellular toxic effect is unlikely because maximal steroidogenesis can be restored with the addition of higher concentrations of angiotensin II and the responses to serotonin and K⁺ are unaffected even by antagonist at a concentration of 2 μ M (Table 2). The decreased cyclic AMP production in the presence of 2 μ M antagonist (Fig. 4) therefore implies that [Sar¹,Ala⁸]angiotensin II not only antagonizes the effect of angiotensin II on glomerulosa cells, but is also independently capable of altering the cyclic AMP output of these cells.

Although it is not possible on present evidence to exclude entirely displacement of endogenously bound angiotensin II as the origin of this effect on basal output, we nevertheless consider it more likely to arise from a modulation of plasma-membrane receptor-adenylate cyclase interactions. Regardless of the mechanism of this effect, the close agreement between the decreases in cyclic AMP and aldosterone production in response to this antagonist is further evidence for the direct involvement of cyclic AMP in the control of aldosterone secretion by angiotensin II and its analogues in glomerulosa cells.

The steroidogenic effect of serotonin and K⁺, both of which stimulate only glomerulosa cells (Müller, 1970; Haning *et al.*, 1970; J. F. Tait *et al.*, 1974; S. A. S. Tait *et al.*, 1974), was unaffected by [Sar¹,Ala⁸]angiotensin II (Table 2). This suggests that the modes of action of serotonin and K⁺ differ significantly from that of angiotensin II.

These studies invoking an intermediary role for cyclic AMP in the mechanism of action of angiotensin II have been facilitated by the use of a sensitive isolated rat adrenal glomerulosa-cell preparation. The effects of the angiotensin II antagonist, [Sar¹,Ala⁸]angiotensin II, have shown that small changes in structure can dramatically alter properties related to steroid and cyclic AMP production. This isolated glomerulosa-cell preparation is therefore a useful system for the study of the mechanisms of action and interactions of the various aldosterone stimulators.

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