Inhibition of Aldosterone Biosynthesis by Atriopeptins in Rat Adrenal Cells

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SUMMARY. The effect of synthetic atriopeptins on basal and stimulated aldosterone secretion was determined in isolated adrenal glomerulosa cells of the rat. Neither atriopeptin I (1-21) or III (1-24, i.e., the Phe-Arg-Tyr carboxy-terminal extension of atriopeptin I) altered basal aldosterone release. However, if the cells were prepared from adrenals of sodium-depleted rats, the basal aldosterone release was increased by 9-fold, compared with cells from normal rats. This elevated release was inhibited by 32% by atriopeptin I and atriopeptin III. Atriopeptin III was more potent than atriopeptin I. Angiotensin II and adrenocorticotropin stimulated the release of aldosterone in a concentration-related manner. Both atriopeptin I and atriopeptin III inhibited the stimulation by the peptides. Atriopeptin I inhibited angiotensin II- and adrenocorticotropin-induced aldosterone production by 50% at concentrations of 12 and 11 nM, respectively, and 0.5 and 0.2 nM, respectively, for atriopeptin III. Potassium-stimulated aldosterone production was also inhibited by atriopeptin I and atriopeptin III with 50% inhibition at concentrations of 10 and 0.4 nM, respectively. Shorter peptides (1-20, 1-19, and 3-19) were equipotent to atriopeptin I (1-21) as inhibitors of angiotensin II-induced steroidogenesis. To determine the site at which atriopeptins inhibit aldosterone synthesis, we used cyanoketone to inhibit 3β-hydroxy-dehydrogenase and dissociate the early and late pathways. Angiotensin II (2 nM) increased the synthesis of pregnenolone (early pathway), as well as the conversion of [3H]corticosterone to [3H]aldosterone (late pathway). Atriopeptin III inhibited basal pregnenolone synthesis by 36% and completely blocked angiotensin II-stimulated synthesis. The peptide similarly inhibited the late pathway. In cells prepared from the zona fasciculata, adrenocorticotropin stimulated the production of corticosterone in a concentration-related manner. However, unlike aldosterone release from zona glomerulosa cells, atriopeptin I and atriopeptin III failed to alter corticosterone release from zona fasciculata cells. The data indicate that atriopeptins are potent and specific inhibitors of aldosterone synthesis. They act at both the early and late pathways of aldosterone synthesis. Unlike vascular smooth muscle, the adrenal cell responds to shorter fragments of the peptide which lack the phenylalanine-arginine carboxy-terminus including the 3-19 ring structure. (Circ Res 57: 113-118, 1985)

MAMMALIAN atria have been found to contain specific granules which resemble the secretory granules found in cells that secrete polypeptide hormones (Kisch, 1956; Jamieson and Palade, 1964; Huet and Cantin, 1974; deBold et al., 1978). Atrial extracts possess biological activity, having natriuretic, diuretic, and vasodilatory properties (deBold et al., 1981; deBold, 1982a; Trippodo et al., 1982; Keeler, 1982; Currie et al., 1983). The active principal is thought to reside in the atrial granules (deBold, 1982b; Garcia et al., 1982). The vasodilator-natriuretic atrial peptides have recently been purified and characterized by several groups (Flynn et al., 1983; Currie et al., 1984; Geller et al., 1984b; Kangawa et al., 1984; Misono et al., 1984). They consist of a series of related polypeptides ranging in size from 19 to 33 amino acids, and contain a core sequence of 17 amino acids within a cystine disulfide bridge. Two of the peptides, atriopeptin III (1-24), which had a Phe-Arg-Tyr C-terminus, and atriopeptin II (1-23),* which has a Phe-Arg C-terminus, produce a natriuresis, diuresis, and vasodilation, whereas the atriopeptin I (1-21),* which lacks the Phe-Arg C-terminus, causes natriuresis and diuresis only (Currie et al., 1984; Geller et al., 1984b). It has been suggested that these peptides may mediate the

* Because of the varied nomenclature used by various groups to describe these polypeptides, we arbitrarily have chosen to define the peptides studied as follows: The peptide Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr will refer to the (1-24) amino acid sequence of the peptide called atriopeptin III and will be used as the amino acid sequence for defining shorter peptides. Thus, with this system, the peptide which is missing the C-terminal Phe-Arg-Tyr from atriopeptin III will be referred to as the amino acid sequence (1-21) and will be called atriopeptin I.
natriuresis and diuresis associated with right atrial distention and may be involved in the physiological regulation of salt and water balance (deBold, 1979; deBold et al., 1981).

The atriopeptins seem to produce their natriuretic and diuretic effects by a direct action on the kidney through renal vasodilatation or inhibition of tubular reabsorption or both (Keller, 1982; Sonnenberg et al., 1982; Keller and Azzarolo, 1983). Recent preliminary reports demonstrate that crude atrial extracts and atrial peptides reduced adenocorticotrophic hormone (ACTH) and angiotensin II-stimulated aldosterone secretion (Atarashi et al., 1984; Chartier et al., 1984). The present study was designed to determine (1) the effect of synthetic atriopeptins on aldosterone secretion, (2) the minimum peptide sequence necessary to inhibit steroidogenesis, (3) the site of inhibition of aldosterone biosynthesis by atriopeptins, and (4) the specificity of atriopeptins for inhibiting aldosterone secretion rather than adrenal steroidogenesis.

Methods

Male Sprague-Dawley rats (200–250 g, Holtzman) were used in these studies. The rats were maintained on a diet of Wayne rat chow, which contained 142 mEq/kg of sodium and 290 mEq/kg of potassium, and were given free access to tap water. To sodium deplete the rats, we placed them on a sodium-deficient diet (Nutritional Biochemical Co.), 10 mEq/kg of sodium and 220 mEq/kg of potassium, and were given distilled water to drink for 5 days (Campbell et al., 1979b). After 1 hour of incubation, the cells were removed by centrifugation. The incubation medium was removed, 10 μg of unlabeled aldosterone (Sigma), 0.05 mg/ml of deoxyribonuclease (Sigma), 0.05 mg/ml of ribonuclease (Sigma), and 2 mg/ml of bovine serum albumin (Calbiochem), and were mixed. The tissue then was incubated for 60 minutes in a Dubnoff metabolic shaker at 37°C under 95% O2, 5% CO2 at 60 rpm. At 20-minute intervals, the cells were dispersed by repeated (30 times) pipetting. The resulting cell suspension was washed twice with the cells were removed by centrifugation. The incubation medium was removed, 10 μg of unlabeled aldosterone (Sigma), 0.05 mg/ml of deoxyribonuclease (Sigma), 0.05 mg/ml of ribonuclease (Sigma), and 2 mg/ml of bovine serum albumin (Calbiochem), and were mixed. The tissue then was incubated for 60 minutes in a Dubnoff metabolic shaker at 37°C under 95% O2, 5% CO2 at 60 rpm. At 20-minute intervals, the cells were dispersed by repeated (30 times) pipetting. The resulting cell suspension was washed twice with

One milliliter of the cell suspension was incubated for 60 minutes in a metabolic shaker at 37°C under an atmosphere of 95% O2, 5% CO2 at 60 rpm. All compounds were added in a volume of 10 μl and an equal volume of the vehicle was added to the control tubes. Atriopeptins (Monsanto) or their vehicle were added to the cells 15 minutes prior to the addition of angiotensin II (Beckman), potassium, or ACTH (Sigma). The atriopeptins were prepared by solid-phase peptide synthesis, and their structures were confirmed by amino acid analysis and verified by gas-phase sequence analysis. Following incubation, the cell suspensions were centrifuged and the supernatant was removed and stored until assayed for aldosterone or corticosterone. Aldosterone and corticosterone were measured by a direct radioimmunoassay (Campbell et al., 1979a; Gomez-Sanchez et al., 1975). The results were expressed as ng/106 cells per hour.

To determine the site of action of atriopeptins on aldosterone biosynthesis, we treated adrenal capsular cells with cyanoketone (WIN 19,579) (1 μM), using the method described by Aguilar and Cah (1979). The drug inhibits 3β-hydroxysteroid dehydrogenase and was used to isolate the early and late portion of the biosynthetic pathway for aldosterone. To determine the effect of atriopeptin II on the conversion of cholesterol to pregnenolone (early pathway), cells were treated with atriopeptin II (31 μM) or its vehicle for 15 minutes before the addition of angiotensin II (2 nm) or its vehicle. After 1 hour of incubation, the cells were removed by centrifugation and the release of pregnenolone into the incubation medium was measured by radioimmunoassay (Bergon et al., 1974). The pregnenolone antiserum was produced in rabbits against the pregnenolone-16-thiopropionate-thyroglobulin conjugate and was provided by Dr. Leon Milewich of the University of Texas Health Science Center at Dallas. The antibody cross-reacted less than 0.007% with 20-hydroxy cholesterol, 22-hydroxy cholesterol, cholesterol, and cyanoketone. The assay consisted of adding 0.3 ml of a 1:36,000 dilution of the anti-pregnenolone serum containing 3000 counts/min of [3H]pregnenolone in phosphate-buffered saline containing 0.1% polyvinylpyrrolidone, pH 7.4, to 0.005 ml of the unknown and standard. The mixture was incubated for 18 hours at 4°C, and the bound and free pregnenolone was separated with dextran-coated charcoal. Results were expressed as ng pregnenolone/106 cells per hour. To investigate the conversion of corticosterone to aldosterone (late pathway), cells were incubated with [3H]corticosterone (50,000 counts/min, 75 Ci:mmol, Amersham) as described for the early pathway. After 1 hour of incubation, the cells were removed by centrifugation. The incubation medium was removed, 10 μg of unlabeled aldosterone and corticosterone were added, and the medium was extracted twice with volumes of ethyl acetate. The extract was dried under N2 and applied to silica gel G thin layer chromatography plates (Whatman LK-5, 250 μm). The plates were developed in dichloromethane: methanol:water:glycerol (150:9:5:0.5:0.4). The migration of aldosterone and corticosterone was visualized with iodine (RF 0.19 and 0.29, respectively), and the corresponding zones were scraped and the radioactivity determined by liquid scintillation spectroscopy (Beckman). The results were expressed as percent corticosterone converted to aldosterone/106 cells per hour.

Statistical differences were determined by analysis of variance and Student’s t-test (Zar, 1974).
Campbell et al./Atriopeptins and Aldosterone

Results

Figure 1 illustrates the effects of atriopeptin I and III on basal aldosterone secretion. In cells from rats on a normal sodium diet, neither atriopeptin I or III altered basal aldosterone release over the range of concentrations tested. In cells from sodium-depleted rats, the basal aldosterone release was increased 9-fold over the release from cells of normal rats. Both atriopeptin I and III reduced this elevated basal aldosterone release in a concentration-related manner. The threshold concentration that produced a significant reduction in aldosterone release was 0.03 nM with both peptides (P < 0.05). Atriopeptin III was a more potent inhibitor than atriopeptin I. Neither peptide reduced the aldosterone release to levels observed in cells from normal animals, but maximally reduced aldosterone by 32%.

Angiotensin II stimulated aldosterone secretion in a concentration-related manner (Fig. 2). Both atriopeptin I and III inhibited the steroidogenic responses to angiotensin II, as indicated by a shift to the right in the angiotensin II concentration-response curve. Atriopeptin III was more effective than atriopeptin I. ACTH stimulation of aldosterone secretion was also inhibited by the atriopeptins. As with angiotensin II, the atrial peptides shifted the concentration-response curve to ACTH to the right.

Figure 3 shows the effect of various concentrations of atriopeptin I and III on stimulated aldosterone release. Angiotensin II (20 nM) increased aldosterone release by 4.3-fold (P < 0.001). There was a concentration-dependent inhibition of this release by atriopeptin I and III with ID50 levels of 12 and 0.5 nM, respectively. Aldosterone secretion was increased 11-fold by ACTH (10 μU/mL) and 6.8-fold by potassium (10 mM) and was inhibited by the atriopeptins in a concentration-related manner. The ID50 levels for inhibition by atriopeptin I of ACTH and potassium were 11 and 10 nM, respectively and were 0.2 and 0.4 nM, respectively, for atriopeptin III.

Peptides shorter than atriopeptin I (1-21) were also effective inhibitors of angiotensin-stimulated aldosterone release (Fig. 4). Peptides with amino acids removed from the carboxy terminus, 1-20 and 1-19, had ID50 levels similar to atriopeptin I (1-21). Similar activity was noted with the 3-19 peptide, which preserves only the ring structure.

Additional studies were performed to determine the site of inhibition of aldosterone biosynthesis by
atriopeptins. Cells were incubated in the presence of cyanoketone to block the 3β-hydroxysteroid dehydrogenase and isolate the early as well as the late pathways to aldosterone synthesis (Aguilera and Catt, 1979). Angiotensin II stimulated both the early and late pathways at a 2 nm concentration (Fig. 5). There was a significant increase in pregnenolone production and an increased conversion of \(^{3} \text{H}\)corticosterone into \(^{3} \text{H}\)aldosterone. Atriopeptin III reduced the basal production of pregnenolone by 36% and completely blocked the ability of angiotensin II to stimulate pregnenolone synthesis. A similar result occurred on the late pathway.

To determine the specificity of atriopeptin inhibition of adrenal steroidogenesis, the effect of atriopeptins on ACTH-stimulated corticosterone production was investigated in zona fasciculata cells. ACTH stimulated corticosterone secretion in a concentration-related manner (Fig. 6, left). Pretreatment with atriopeptin III did not alter this concentration-response relationship. Furthermore, when a range of concentrations of atriopeptin I and III were tested, neither peptide altered ACTH-induced corticosterone release (Fig. 6, right). Thus, atriopeptins inhibited ACTH-induced aldosterone release in zona glomerulosa cells, but not ACTH-induced corticosterone release from zona fasciculata cells.

**Discussion**

The results of this study show that atriopeptins are potent and selective inhibitors of aldosterone biosynthesis. The peptides did not alter basal or unstimulated aldosterone release in cells from rats maintained on a normal sodium diet. However, if aldosterone release was stimulated by angiotensin II, ACTH, or potassium, they inhibited release in a concentration-related manner. Furthermore, the concentrations of the atriopeptin required to inhibit the stimulated aldosterone release were similar, whether angiotensin II, ACTH, or potassium was the stimuli. Also, atriopeptin III was consistently more potent than atriopeptin I as an inhibitor with an \(\text{ID}_{50}\) of 0.2-0.5 nm for atriopeptin III and 10-12 nm for atriopeptin I. The basal release of aldosterone was increased, if adrenal cells were prepared from sodium-depleted rats, compared to cells from rats on a normal sodium diet. The atriopeptins also inhibited this type of stimulated aldosterone release but by only 32%. The reason for their limited effectiveness in sodium-depleted rat cells is unknown but it is possible that some stimuli may be resistant to the inhibitory effects of the atriopeptins. Also, the atriopeptins appear to inhibit aldosterone release selectively. In contrast to the results in zona glomerulosa cells, the peptides did not inhibit ACTH-induced corticosterone release from zona fasciculata cells. Thus, they are not general inhibitors of aldosterone synthesis but are selective inhibitors of aldosterone synthesis that has been stimulated.

Atarashi and coworkers (1984) reported that crude extracts of rat atria inhibited the basal release of aldosterone as well as release stimulated by ACTH or angiotensin II, and Chartier et al. (1984) reported that atrial peptides inhibited stimulated aldosterone release. In general, our findings are in agreement, and indicate that atriopeptins have inhibitory activity similar to that observed in the atrial extracts. It should be noted that the atrial extracts reduce basal aldosterone release whereas atriopeptins do not. However, the basal release reported by Atarashi et al. (1984) was 5-6 times greater than we observed, and may represent aldosterone release stimulated by a different sodium diet or by the higher potassium content of the cell isolation medium (5.4 mEq/liter vs. 0 mEq/liter for the present study) or incubation medium (5.4 mEq/liter vs. 4.5 mEq/liter for the present study). Considering these differences, it is not surprising that the atrial extract reduced aldosterone release under the basal conditions described, whereas atriopeptins did not, under a different set of conditions. Also, we point out that
Antiopeptins I and III may not be the naturally occurring peptides released by the atria. High and intermediate molecular weight precursors of the antiopeptins have been identified, and these peptides may prove to be the natural cleavage products (Geller et al., 1984a; Kangawa et al., 1984). The effect of these larger polypeptides on aldosterone secretion is not known, but may account for the differences observed between atrial extracts and synthetic antiopeptins.

The present studies provide some insights into the mechanism of inhibition of aldosterone release. Since the peptides inhibit angiotensin II-, ACTH-, and potassium-stimulated aldosterone release, it is unlikely that the antiopeptins block the receptors on the adrenal cell for these hormones. Rather, they must inhibit at a common pathway distal to the hormone receptor. Angiotensin II, ACTH, and potassium stimulate the synthesis of pregnenolone from cholesterol (the early pathway), and potassium and high concentrations of angiotensin II also stimulate the conversion of corticosterone to aldosterone (the late pathway) (Muller, 1971; McKenna et al., 1978; Aguilera and Catt, 1979). Antiopeptin III blocks the effects of angiotensin II on both the early and late pathways of aldosterone biosynthesis. The ability to block both of the regulated steps in aldosterone biosynthesis may account for the ability to inhibit a variety of stimuli.

A series of peptides were studied to determine the minimal peptide sequence which was necessary for this biological activity. Antiopeptin III (1-24) was the most potent inhibitor tested and was approximately 30 times more potent than antiopeptin I (1-21). Further shortening of the carboxy terminus (1-20 and 1-19) did not reduce biological activity further. Also, shortening the peptide from the amino terminus so that only the ring structure was maintained (3-19) did not further alter activity. Thus, the 3-19 sequence is adequate to inhibit aldosterone release. On intestinal smooth muscle, antiopeptin I, II, III, and the 3-19 fragment produced relaxation (Currie et al., 1984; Geller et al., 1984b). Thus, the adrenal cortex and intestinal smooth muscle seem similar in their structural requirements for biological activity. However, in the renal vasculature and rabbit aorta, antiopeptin II, III, and larger peptides produce vasodilation, whereas antiopeptin I and shorter peptides are inactive (Currie et al., 1984; Geller et al., 1984; Oshima et al., 1984). This would indicate that the Phe-Arg structure must be maintained on the carboxy terminus of the peptide for vasodilatory activity.

Finally, the significance of antiopeptin inhibition of aldosterone secretion is unknown. However, most potent diuretics that are used as pharmacological agents cause a natriuresis and diuresis which is associated with activation of the renin-angiotensin-aldosterone system (Vander and Carlson, 1969; Oh and Carroll, 1978; Swartz et al., 1980; Jackson et al., 1982). The stimulation of aldosterone secretion by angiotensin increases the renal excretion of potassium and results in hypokalemia (Oh and Carroll, 1978; Swartz et al., 1980; Jackson et al., 1982). The hypokalemia is a major side effect encountered with these drugs. The diuretic-induced potassium loss and hypokalemia can be corrected by inhibiting the renal actions of aldosterone. This has been accomplished by inhibiting renin release with β-adrenergic antagonists (Oh and Carroll, 1978), inhibiting angiotensin II synthesis with converting enzyme inhibitors (Swartz et al., 1980), or inhibiting the renal receptor with the antagonist spironolactone (Jackson et al., 1982). Antiopeptins combine in one molecule a potent natriuretic and diuretic agent with an inhibitor of angiotensin II-induced aldosterone secretion. As a result, these compounds may induce a natriuresis without potassium loss or hypokalemia, and thus be superior to the available pharmacological agents.

Note Added in Proofs

Since the submission of this manuscript, Goodfriend et al. (1984) reported in isolated bovine adrenal cells that antiopeptin III inhibited aldosterone secretion stimulated by angiotensin II, potassium, and dibutyryl cyclic adenosine monophosphate (cAMP). We had similar results with angiotensin II and potassium in isolated cells from the rat adrenal. However, in contrast to our findings, antiopeptin III also inhibited basal aldosterone release. This discrepancy may be due to the methodological differences pointed out above in the discussion or to species differences. Goodfriend et al. reported that angiotensin II stimulated the early pathway of aldosterone biosynthesis, and this stimulation was inhibited by antiopeptin III. However, the antiopeptin did not alter the conversion of 25-hydroxy cholesterol or progesterone to aldosterone. Thus, the peptide did not alter the late pathway under basal conditions. The effect of the antiopeptin on angiotensin stimulation of the late pathway was not determined. In the present study, antiopeptin III inhibited the effect of angiotensin II on both the early and late pathways to aldosterone biosynthesis.

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