Properties of Angiotensin II Receptors in the Bovine and Rat Adrenal Cortex

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SUMMARY

Receptors for angiotensin II have been identified and characterized in bovine and rat adrenal cortex by binding studies with tritiated and monoiodinated angiotensin II. The angiotensin II binding sites of bovine adrenal cortex homogenate were enriched severalfold in a microsomal membrane fraction together with alkaline phosphatase, adenylate cyclase, 5'-nucleotidase and 21β-hydroxylase. Uptake of angiotensin II by adrenal cortex particles and adrenal cells was rapid, reaching equilibrium at 15 to 30 min in the presence of 0.2 to 1.5 nM angiotensin II. The equilibrium association constant of angiotensin II for bovine adrenal cortex receptors was 0.5 nM at 22°C, and that for rat adrenal particles was significantly higher. Dissociation of bound angiotensin II from adrenal particles and cells was also rapid, with initial half-time of 13 to 23 min. Angiotensin II released from adrenal cortex binding sites at low pH retained activity in subsequent binding studies, whereas angiotensin II in the incubation medium was rapidly inactivated. Degradation of angiotensin II by adrenal cortex particles was partially inhibited by addition of unrelated peptides including glucagon and insulin, by reducing agents such as dithiothreitol, and by reduced temperature.

Fragments and analogues of angiotensin II showed binding-inhibition potencies which correlated with their biological activities in vivo. In particular, competitive antagonists, such as the (Sar1, Ala2) derivative of angiotensin II, inhibited angiotensin II binding in proportion to their antagonistic activity in vivo. This system provides a simple and rapid method for evaluation of the competitive binding activity of angiotensin agonists and antagonists in vitro.

In contrast to angiotensin II, the decapeptide angiotensin I exhibited relatively low affinity for angiotensin II binding sites in competitive studies, and direct binding studies with monoiodinated 125I-angiotensin I showed considerably lower uptake than that of angiotensin II. The uptake of labeled angiotensin I and II by adrenal medulla homogenates was much lower than that of adrenal cortex particles, and again angiotensin II showed higher binding affinity than angiotensin I.

These findings illustrate the presence of high affinity specific binding sites for angiotensin II in bovine and rat adrenal cortex and suggest a plasma membrane location for the angiotensin II receptors.

Angiotensin II is a potent stimulus to the secretion of aldosterone by the adrenal cortex (1-5), and earlier studies have shown uptake of tritiated angiotensin by rat adrenal glands in vivo (6). The exact site of the receptors for angiotensin II in its target cells has not been defined, although adrenal mitochondria have been reported to bind labeled angiotensin II in vitro (7, 8) and cardiac muscle nuclei have been shown to contain radioactivity soon after in vivo administration of labeled angiotensin II to rats (9). In vascular smooth muscle, convincing evidence for the presence of angiotensin II receptors in the cell membrane has been obtained (10). Previous studies on angiotensin II binding by adrenal cortex fractions have employed iodinated angiotensin II containing a proportion of the inactive diiodinated peptide (7) and have indicated that the major binding site may lie in the mitochondria (8); also, the binding of angiotensin I was sometimes greater than that of angiotensin II (11).

To further define the location and binding properties of the adrenal cortex receptors for angiotensin II, quantitative uptake studies have been performed on subcellular fractions of the bovine and rat adrenal glands. These experiments were performed with both monoiodinated 125I-labeled angiotensin and tritiated angiotensin, and have demonstrated that the adrenal receptor sites consistently display more active binding of angiotensin II than of angiotensin I. In addition, the specific binding sites for angiotensin II are situated in a microsomal cell fraction with characteristics of plasma membrane, consistent with a primary interaction of angiotensin II with receptors in the adrenal cell membrane.

EXPERIMENTAL PROCEDURE

Materials

Tritiated (Aom1, Val3) angiotensin II was obtained from the Commissariat a l'Energie Atomique (Saclay, France) and had a stated specific activity of 40 Ci per mmole. Determination of the specific activity by liquid scintillation counting and radioimmunoassay of the peptide gave a value of 67 Ci per mmole. As this value exceeds the theoretical maximum of 58 Ci per mmole obtainable by tritiation of the tyrosyl residues alone, it is likely that additional tritiation may have occurred at the histidine residue during preparation of the labeled peptide. The monoiodinated 125I-labeled peptides were prepared by Schwarz-Mann, Orangeburg, N. Y. The specific activity of

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the [3H]-angiotensin II was determined by radioimmunoassay and was usually in the range of 900 to 1000 μCi per μg (1000 Ci per mmole); moniodination was confirmed by pronase digestion and chromatography of the labeled products. Labeled peptides were stored as frozen aliquots at −16° and were used only once after thawing. The (Sar1,Ala8) analogue of angiotensin II was a gift from Dr. A. W. Castellion (Norwich Pharmacal Co., Norwich, N. Y.); (Val7) angiotensin II peptides were generously provided by Dr. Riniker (Ciba Chemical Co.); and all other peptides were obtained from Schwarcz-Mann, Orangeburg, New York.

**Methods**

**Preparation of Subcellular Particles—**Bovine adrenals were obtained within 10 min after death, immediately sliced into 0.3- to 0.5-cm sections, and kept in ice-cold Krebs-Ringer-phosphate buffer (pH 7.4) containing 0.2% glucose and 1% bovine serum albumin. After removal of the medulla, the cortex was dissected from the capsule and minced into small pieces. Minced tissue was washed twice with ice-cold Krebs-Ringer-phosphate buffer, drained, and homogenized with Medium A (20 mM sodium bicarbonate) in a large Dounce homogenizer with 10 strokes of the loose pestle. Twenty milliliters of Medium A per 1 g wet weight of minced tissue were used. The homogenate was stirred for approximately 15 min and subsequently filtered through coarse fiberglass screen and nylon gauze. The filtered homogenate was spun at 1,500 × g for 10 min. Combined supernatants were spun at 20,000 × g for 30 min. The 20,000 × g pellet was washed once with Medium A (4 ml per g wet weight of starting material) and kept on ice. For further purification and removal of the majority of mitochondria, the 20,000 × g pellet was resuspended by gentle homogenization in 0.25 M sucrose buffered with Medium B (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, disodium salt, pH 7.2). One milliliter of 0.25 M sucrose in Medium B was used for 1 g wet weight of starting material. The resuspended pellets were layered on a discontinuous sucrose gradient (31.5, 38.5, and 42.5%; w/w) sucrose in Medium B) and spun for 120 min in a Beckman SW 25.1 rotor at 25,000 rpm. After sucrose gradient centrifugation, the layers were collected by aspiration, diluted 20-fold with Medium A, and sedimented at 40,000 × g for 40 min. All steps were performed between +2° and +4°.

**Binding Assay—**Aliquots of freshly prepared particulate fractions (0.1 to 0.8 mg of protein) were incubated in glass tubes (12 × 75 mm) in assay buffer (120 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.2% serum albumin) with labeled angiotensin II in a final volume of 0.1 to 0.2 ml. After a given time the contents of the tubes were rapidly diluted with 4 ml of ice-cold assay buffer (albumin omitted) and filtered through Millipore nitrocellulose HAWP (0.45 μ) filters. Two rinses (4 ml each) of the assay tubes were employed to wash the filters prior to determination of trapped radioactivity. Appropriate controls were run to determine trapping or adsorption of labeled angiotensin to Millipore filters in the absence of receptor. This was achieved by performing the same sequence of operations as described above, with omission of binding particles from the assay mixture. The quantity of radioactivity found on Millipore filters in these control experiments was subtracted from that found by filtering assay mixtures containing subcellular particles; the difference is referred to as the bound angiotensin. Radioactivity on Millipore filters was determined by counting in a liquid scintillation spectrometer for binding studies with [3H]-angiotensin II. Filters were immersed in either 10 ml of BBS-3 solution (Beckman) or in Aquasol (New England Nuclear). The tritium counting efficiency under these conditions was between 30 and 35%. When [3H]-angiotensin was used, Millipore filters were dried and counted in an automatic gamma spectrometer with a counting efficiency of 50% for [3H]. Binding constants were calculated by computer fitting as previously described (12).

**Enzyme Measurements—**Adenylate cyclase was measured according to Krishna (13) as modified by Ramachandran (14). The assay medium contained 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM ATP, 0.5 mM cyclic AMP, 10 mM MgCl2, 30 mM KCl, 10 mM theophylline, and 20 mM phosphocreatine. Each assay tube (final volume, 0.125 ml) contained 1 μCi of [α-32P]ATP, 12 μg of creatine phosphokinase, and 50 μg of bovine serum albumin. 5'-Nucleotidase was determined as described by Michell and Hawthorne (15); alkaline phosphatase and succinic cytochrome c reductase were determined as previously described (12). 21β-Hydroxylase was measured as reported by Sater et al. (16).

**Results**

**Binding Assay—**As described under “Methods,” particle-bound hormone was separated from free hormone by Millipore filtration. By counting the radioactivity left in test tubes after washing and filtration, it was found that only negligible amounts of labeled hormone remained in the washed test tubes. Radioactivity on Millipore filters obtained by filtering the assay mixture without receptor (control value) was a constant proportion of the total radioactivity present in the test tube prior to filtration (0.8 to 1%). These control values were unchanged by the presence of 1 to 5 × 10^-6 M unlabeled angiotensin II in the assay. The presence of excess unlabeled angiotensin II (1 to 5 × 10^-4 M) in assay mixtures containing particulate receptors reduced the radioactivity found on Millipore filters to the control value (Table I).

**Uptake and Degradation of Labeled Angiotensin by Bovine Adrenal Subcellular Particles—**Subcellular particles from bovine adrenal cortex took up tracer angiotensin II in a time- and temperature-dependent manner. At 22 and 37°, the time course of uptake as shown in Fig. 1 was commonly observed. It is apparent that after 2 min (the first measured point) no increase

**Table I**

| Tracer | Radioactivity in assay tube after filtration | Radioactivity found on Millipore filters after filtration |
|--------|--------------------------------------------|--------------------------------------------------------|
|        |                                   | + receptor        | + receptor + 4 μg unlabeled angiotensin II | - receptor        |
| [3H]Angiotensin II | 12,200 | 4,520 ± 280 | 120 ± 35 | 112 ± 12 |
| [3H]-Angiotensin II | 11,000 | 4,470 ± 82 | 110 ± 20 | 180 ± 8 |
FIG. 1. Uptake of \([^3\text{H}]\text{angiotensin II (1.2 nM) by a 1500 } \times g\) particulate fraction from bovine adrenal cortex (0.7 mg of protein) at 22 and 37°. The final concentration of glucagon was 0.3 mg per ml and that of dithiothreitol was 5 mM.

Fig. 2. Examination of "bound" and "free" \[^3\text{H}\]angiotensin II by thin layer chromatography. Free angiotensin II and particle-bound (and acid-released) angiotensin II from a similar experiment as shown in Fig. 3 were applied on thin layer cellulose plates (Eastman 6064) and developed with sec-butyl alcohol-3% ammonia (105:35). Developed chromatograms were scanned for radioactivity (Packard Radiochromatogram Scanner, model 7201). The positions of angiotensin II (Val^6) peptides and free tyrosine in this system are indicated by arrows. The abbreviations refer to angiotensin II peptides missing either one, two, three, or four amino acids from the NH_2-terminus (C7-C4) or one or four amino acids from the COOH-terminus (N7, N4). Note that the presence of glucagon (0.3 mg per ml) and dithiothreitol was 5 mM in experiments with \[^3\text{H}\]angiotensin. Only dithiothreitol (5 mM) was present in experiments with \[^125\text{I}\]angiotensin. This proteolytic process was inhibited by lowering the temperature (12°), by the addition of several peptides including glucagon and insulin, and by sulfhydryl-protecting agents such as dithiothreitol (Figs. 1 to 3). With glucagon (0.3 mg per ml) and dithiothreitol (5 mM), angiotensin degradation was minimized and a steady state value was reached after 10 to 15 min at 22° and after 20 to 30 min at 12° when angiotensin II was 0.2 to 1.5 nM. The steady state value reached at 12° was 30% lower than at 22° and this was independent of concentrations of receptor protein in the assay between 40 and 450 µg (Fig. 4).

The term "steady state" is used here as defined by Rodbell et al. (18).

Fig. 3. Rebinding of \[^3\text{H}\]- and \[^125\text{I}\]-angiotensin after exposure to bovine adrenal cortex subcellular particles. Both traces were incubated with adrenal cortex subcellular particles (2.0 mg of protein from a washed 20,000 } \times g sediment) in assay buffer for 30 min at 22° (final volume, 650 µl). After centrifugation at 20,000 } \times g for 30 min at 4°, supernatants were aspirated and saved on ice. The pellets were quickly resuspended in ice-cold assay buffer and centrifuged as above. Radioactivity in combined supernatants and pellets was counted, and bound and free angiotensin were found to add up to the total amount added prior to incubation. Pellets were resuspended in 0.5 ml ice-cold 0.05 M acetic acid (pH 3.2) and centrifuged as above. Ninety-seven per cent of the previously particle-bound radioactivity was eluted in the supernatant. The acetic acid eluate then was neutralized with 0.1 M NaOH. Supernatants and the neutralized eluate from particles were tested for rebinding using fresh particles and Millipore filtration. Mean values for four experiments are shown (± S.D.). Glucagon was 0.3 mg per ml and dithiothreitol was 5 mM in experiments with \[^3\text{H}\]angiotensin. Only dithiothreitol (5 mM) was present in experiments with \[^125\text{I}\]-angiotensin. Note that the initial incubation time (30 min) was longer than the time at which a steady state value for uptake is reached at 22° (10 to 15 min).

Fig. 4. Bound \[^3\text{H}\]angiotensin II (steady state values) as a function of receptor protein concentration and temperature. Glucagon (0.3 mg per ml) and dithiothreitol (5 mM) were present in assay mixtures. Each point is a mean value from duplicate determinations.

The term "steady state" is used here as defined by Rodbell et al. (18).
Addition of Unlabeled Angiotensin II (to 1.25 mM)

Addition of Unlabeled Angiotensin II to bovine adrenal subcellular particles (○). The assay mixture (final volume, 0.12 ml) contained the 20,000 × g particulate fraction (0.4 mg of protein) from bovine adrenal cortex, glucose (0.3 mg per ml), dithiothreitol (5 mM), and ethylenediamine tetraacetic acid (1 mM). Each point is a mean value from duplicate determinations. In a parallel experiment unlabeled angiotensin II in 10 μl (final concentration, 1.5 μM) was added (arrow) and the exchange of bound tracer with unlabeled angiotensin II was followed (○). The half-time of dissociation (18 to 23 min) in this and similar experiments was estimated by fitting binding data to the pseudo-first order rate equation and used to calculate the dissociation rate constant as given in Table III. B, association rate constant determination for uptake of [3H]angiotensin by bovine adrenal cortex subcellular particles. Binding data from the experiment described in Fig. 5A are fitted to the second order rate equation. N, total number of binding sites estimated from Scatchard plots (750 fmoles per mg of protein); H, total hormone added at zero time; H₀, amount of hormone bound at time t. 

Addition of excess unlabeled angiotensin II after a steady state was reached caused rapid release of the bound radioactivity from bovine adrenal cortex particles (Fig. 5).

Subcellular Fractionation and Localization of Angiotensin II Receptors in Microsomal Fraction of Adrenal Cortex—More than 90% of the angiotensin II binding of a filtered adrenal cortex homogenate could be recovered in the 1,500 × g sediment, and the 20,000 × g sediment of the 1,500 × g supernatant (Table II). Since the receptor concentration (estimated by Scatchard plots) was 2 times higher in the 20,000 × g than in the 1,500 × g sediment, it was chosen as the starting material for further purification.

After discontinuous sucrose gradient centrifugation (see "Methods"), two prominent bands of particulate material collected at the top of the 31.5 (w/w) sucrose layer and at the 31.5/38.5 (w/w) layer interface. The former fraction (Fraction I, Table II) was shown to be a predominantly vesicular fraction when examined by electron microscopy, while the latter (Fraction II, Table II) was mainly composed of mitochondria.

Angiotensin II binding sites were 2- to 3-fold enriched in Fraction 1 compared to the 20,000 × g sediment, whereas Fraction II (mitochondria) showed less angiotensin II binding than the starting material. In agreement with the morphological characteristics, Fraction 2 was markedly enriched in sucinic cytochrome c reductase, while Fraction 1 was enriched in alkaline phosphatase, adenylate cyclase, 21β-hydroxylase, and 5'-nucleotidase. The activity of 5'-nucleotidase (a marker enzyme for liver cell membranes) was low in the filtered homogenate of the bovine adrenal cortex and was inhibited 30 to 50% by sodium potassium tartrate (10 mM), indicating a significant contribution of acid phosphatase to 5'-AMP hydrolysis. Alkaline phosphatase has activity against 5'-AMP at pH 7.4 (20) and the true contribution of a 5'-nucleotidase to 5'-AMP hydrolysis in different subcellular fractions of bovine adrenal cortex remains to be established. This has been noticed by others (16). Since angiotensin II receptors in the 20,000 × g pellet from bovine adrenal cortex showed the same properties (affinity constants and apparent affinities of angiotensin analogues) as the purer Fraction 1 after sucrose gradient centrifugation, the former was utilized for most of the binding studies. It was then possible to complete binding studies employing both [3H]- and [125I]-angiotensin II within 12 hours after obtaining bovine adrenal glands. The subcellular distribution of the angiotensin II receptor sites suggested that these sites are located on the plasma membrane. The affinity of angiotensin II II receptors from bovine adrenal cortex II was calculated to be 0.5 nM⁻¹ at 22°C using either [3H]angiotensin II (Fig. 5A) and [125I]angiotensin II (Table III). In one series of experiments at 12°C with [3H]angiotensin, Kᵢ was calculated to be 0.2 nM⁻¹. This suggests a temperature dependence of the association constant and may explain the lower steady state binding at 12°C compared to 22°C (Fig. 4). While the number of high affinity sites and their respective affinity constants demonstrated only small variations when different receptor preparations were compared, the number of low affinity sites and their affinity constants showed more marked variation (Table III). This was found to be due to changes which occurred in the receptor preparation during isolation and storage. Freshly isolated receptor preparations did not always display the lower affinity sites (Table III). After 4 to 5 hours of standing at 2°C, a slight decrease in the number of high affinity sites (about 10 to 20%) was observed, accompanied by an appearance of the second, low affinity site. This process could be markedly enhanced by freezing and thawing. A freshly isolated 1500 × g sediment from bovine adrenal cortex had only one (high affinity) site (Kᵢ = 0.35 nM⁻¹, Nᵢ = 304 fmoles per mg of protein). After quick freezing, storage in liquid nitrogen for 24 hours and thawing, Kᵢ = 0.4 nM⁻¹, Nᵢ =

Alkaline phosphatase can be demonstrated in purified cell free suspensions from bovine adrenal cortex.
TABLE II
Subcellular distribution of angiotensin II receptors and enzyme activities in bovine adrenal cortex

Enzyme activities are expressed in micromoles per mg of protein per hour at 37°C, except for adenylyl cyclase which is in picomoles per mg of protein per 15 min at 37°C. Binding of angiotensin II is given as the concentration of high affinity sites (femtomoles) per mg of protein, as determined with [3H]angiotensin from Scatchard plots for each fraction at 22°C. The values in parentheses are relative specific activities compared to the original homogenate (=1).

Bovine adrenals were collected in 20 mM Tris-HCl buffer, 150 mM NaCl, 4 mM KCl, and 0.2% glucose but otherwise the experiment was performed as described under "Method." 5'-Nucleotidase in all fractions was measured in the presence of 10 mM sodium potassium tartrate. Liberation of phosphate from 5'-AMP in the homogenate was 1.5 to 2 times higher when sodium potassium tartrate was absent. N.D., not determined.

| Fraction                          | Protein | Angiotensin II binding | Alkaline phosphatase | 5'-Nucleotidase | Succinic cytochrome c reductase | 21β-Hydroxylase (11-deoxycortisol formed) | Adenylyl cyclase (10 mM NaF) |
|----------------------------------|---------|------------------------|---------------------|----------------|-------------------------------|----------------------------------------|-----------------------------|
|                                  | mg      | fmole/mg protein       | %                   | %              | %                            | %                                      | %                           |
| Filtered homogenate              | 1,440   | 130                    | 100                 | 1.3 (1)        | 0.07 (1)                      | 4.5 (1)                                | 100                         |
| 1,500 X g sediment               | 180     | 12.5                   | 340                 | 32             | 3.0 (2.3)                     | 20 (2.6)                               | 63                          |
| 1,500 X g supernatant            | 1,000   | 70                     | N.D.                | 1.3 (1)        | 0.06 (0.9)                    | 4.2 (0.9)                              | 63                          |
| 20,000 X g sediment              | 170     | 12                     | 600                 | 66             | 3.5 (2.7)                     | 32 (3.4)                               | 41                          |
| 20,000 X g supernatant           | 740     | 51                     | N.D.                | 0.6 (0.5)      | 0.02 (0.3)                    | 1.2 (0.3)                              | 15                          |
| Fraction 1 from sucrose gradient | 40      | 2.8                    | 1580                | 34             | 6.3 (5)                       | 0.36 (5.4)                             | 15                          |
| Fraction 2 from sucrose gradient | 65      | 4.2                    | 520                 | 17             | 1.5 (1.2)                     | 0.13 (2.0)                             | 8                           |

Fig. 6. Uptake of [125I]-angiotensin II by isolated bovine adrenal cortex cells. Bovine adrenal cortex cells were isolated by collagenase digestion (22). Cells (equivalent to 200 μg of protein) were preincubated for 5 min at 22°C in Krebs-Ringer-phosphate buffer containing 2% serum albumin and 0.2% glucose. At zero time, [125I]-angiotensin II in albumin was added (22,500 cpm). Final volume was 200 μl. Cells were spun down at times indicated (2 min at 14,000 X g), the supernatant was removed by aspiration, and the pellet was rinsed with 250 μl of ice-cold medium containing 10% (w/v) sucrose. The bottom of the test tube (Brinkmann) was cut off, and radioactivity in the rinsed pellet was counted. In a second series of incubations, 10 μl of unlabeled angiotensin II was added after 10 min (final concentration, 5 X 10^-8 M), and the experiment performed as above. Control values obtained at 2 and 20 min by simultaneous addition of labeled and unlabeled angiotensin II (1,100 cpm) have been subtracted. The same experiment was performed simultaneously with the Millipore technique. The results were identical except that control values were slightly lower in the latter method (770 cpm). Each point is a mean value of three determinations except for those obtained after addition of unlabeled angiotensin, which are means of two determinations.

Fig. 7. Scatchard analysis of steady state binding data at 29°C for bovine adrenal cortex angiotensin II receptors with [3H]angiotensin II.

150 fmole per mg of protein. A second, low affinity site (K1 = 0.014 nM-1, N2 = 5000 fmole per mg of protein) had appeared. Kinetic studies in which uptake and dissociation of [3H]angiotensin II were measured resulted in values for the association constant which are in good agreement with binding constants calculated from steady state studies (Table III).³

³ In addition to the values derived graphically and presented in Table III, rate constants were also determined by computer curve fitting using the integrated second-order rate equation. The values obtained for bovine adrenal cortex particles by this method were: k1 = 3 X 10⁴ s⁻¹ M⁻¹; k2 = 13.3 X 10⁻¹ s⁻¹; equilibrium association constant (K1) = 0.23 nM⁻¹.
These peptides upon responses such as blood pressure or contraction of smooth muscle included glucagon, insulin, adrenocorticotropic hormone, and angiotensin II, and the activities of the 1-7 heptapeptide, 3-8 hexapeptide, 4-8 pentapeptide, and 5-8 tetrapeptide were two or more orders of magnitude below that of the intact octapeptides. Angiotensin I and the synthetic 2-8 heptapeptides of each form were only slightly less active than the intact octapeptide. All angiotensin fragments and analogues (Figs. 9 and 10). The Vale and Ile5 forms of angiotensin II were almost equipotent, and the specificity of the angiotensin II binding sites was indicated by their efficacy as inhibitors of angiotensin II activity in conventional assay systems. The (Phe4, Tyr8) inhibitory analogue of angiotensin II (23) was about 10% as potent as the normal peptide in competing for binding with radioactive angiotensin II at adrenal receptor sites. By contrast, the more effective (Ser4, Ala8) inhibitory analogue (24, 25) displayed almost twice the binding-inhibition potency of angiotensin II, demonstrating a direct effect of the antagonist in blocking the combination of angiotensin II with the specific receptor site in the adrenal cortex (Fig. 10).

**Organ Distribution of Angiotensin II Receptors**—We examined the organ distribution of angiotensin II receptors in rat tissues under the conditions which were found to minimize angiotensin degradation in bovine cortex subcellular fractions (Table IV). It is obvious that the information obtained in these experiments is limited by the effects of angiotensin-degrading systems which may differ from species to species and even from organ to organ in one species. Nevertheless, the rat adrenal demonstrated the highest concentration of receptors per mg of protein among all organs tested in every experiment. It was noted that kidney and liver subcellular fractions demonstrated extreme variations when tested from individual animals rather than mixing organs from different animals. The reason for this variation is unknown to us at this time. Angiotensin II has actions on the adrenal medulla (26), and bovine adrenal medulla had consistently about 10% of the binding site concentration of the adrenal cortex (Figs. 11 and 12). These binding sites had similar affinities for angiotensin II as those found in the adrenal cortex and could be purified in a similar manner.

**Angiotensin I Versus Angiotensin II Binding**—From the biological activity or with inhibitory activity in vivo. The (Phe4, Val5, Tyr8) analogue of angiotensin II was totally devoid of binding-inhibition activity, in keeping with its undetectable biological potency. By contrast, known antagonists of angiotensin II showed competitive binding activity in proportion to their efficacy as inhibitors of angiotensin II activity in conventional response systems. The (Phe4, Tyr8) inhibitory analogue of angiotensin II (23) was about 10% as potent as the normal peptide in competing for binding with radioactive angiotensin II at adrenal receptor sites. By contrast, the more effective (Ser4, Ala8) inhibitory analogue (24, 25) displayed almost twice the binding-inhibition potency of angiotensin II, demonstrating a direct effect of the antagonist in blocking the combination of angiotensin II with the specific receptor site in the adrenal cortex (Fig. 10).

### Table III

**Binding parameters for angiotensin II receptors**

| Adrenal tissue | Tracer | Temperature | High affinity site | Low affinity site |
|---------------|-------|-------------|--------------------|------------------|
|               |       |             | $K_1$ (nM)         | $N_1$ (fmoles/mg protein) |
| Bovine (6)    | [3H]Angiotensin II | 22°C | 0.54 ± 0.2 | 600 ± 140 |
| Bovine (3)    | [3H]Angiotensin II | 12°C | 0.2 ± 0.01 | 788 ± 210 |
| Bovine (5)    | [3H]Angiotensin II | 22°C | 0.5 ± 0.1 | 620 ± 210 |
| Rat (1)       | [3H]Angiotensin II | 22°C | 5.4 | 12.0 |

### A. Steady state data

### B. Kinetic data

| Tissue | Tracer | Temperature | Association rate constant ($k_a$) | Dissociation rate constant ($k_d$) | Equilibrium constant ($k_a/k_d$) |
|--------|-------|-------------|----------------------------------|-----------------------------------|----------------------------------|
| Bovine | [3H]Angiotensin II | 12°C | $2.4 \times 10^5$ M⁻¹ s⁻¹ | $5 \times 10^{-4}$ s⁻¹ | $4.8 \times 10^6$ M⁻¹ |

The affinity constant (0.5 nM⁻¹) for angiotensin II is rather low compared to the levels of the hormone necessary to stimulate aldosterone secretion in vivo. We therefore tried in several experiments at 22°C to demonstrate sites with much higher affinity by lowering the [3H]angiotensin II concentration to 80 pM and that of [3H]-angiotensin II to 2.5 pM. The results of these studies, when analyzed by Scatchard plots, were scattered around a value of (bound to free) which could be predicted by $(K_1 \times N_1) + (K_2 \times N_2)$. This indicates that the concentration of sites with higher affinity must be much less than that of the sites characterized above.

Our failure to detect sites with higher affinity in bovine adrenal cortex preparations cannot be attributed to the techniques employed here. We had no difficulties in demonstrating these sites in rat adrenal subcellular particles (Fig. 8).
Fig. 8. Angiotensin II receptors in rat adrenal tissue. Whole rat adrenals were homogenized as described under "Methods" for bovine adrenal cortex and a 20,000 X g (30 min) particulate fraction was prepared from the 1,500 X g (10 min) supernatant of the filtered homogenate. A displacement curve (using [123I]-angiotensin II at 0.1 nM (A) and a Scatchard plot of the steady state binding data (B) are shown. (T = 22°; assay volume, 0.2 ml; 0.32 mg of protein per sample.)

Notes: 1. Equilibrium constant of the high affinity site is 10 times higher than that in bovine adrenal cortex. Sar9, Ala10-angiotensin II is referred to in the text.

Competitive binding results given above, it was evident that the decapetide angiotensin I had low affinity for the angiotensin II receptors of bovine adrenal cortex. This was confirmed when direct binding of [123I]-angiotensin I was tested with different bovine adrenal subcellular fractions (Fig. 11).

High affinity, saturable binding of [123I]-angiotensin I was much lower in the bovine adrenal cortex than that of [123I]-angiotensin II, and binding-inhibition experiments indicated that angiotensin II rather than angiotensin I competed for the binding sites (Fig. 12). This suggested that conversion of angiotensin I to angiotensin II occurred in washed bovine adrenal cortex particles. Such conversion was confirmed by radioimmunoenzyme and thin layer chromatography of free angiotensin and peptide released from binding particles at pH 3.5.

Thus, under the conditions employed here, it has not been possible to confirm earlier reports (11) that angiotensin I rather...
Male Sprague-Dawley rats (250 g) were killed by decapitation and various organs were removed and immediately homogenized after mincing into small pieces in ice-cold NaHCO₃ (20 mM) as described for bovine adrenal cortex. The homogenate was stirred for 10 min at 0°C, filtered, and centrifuged for 30 min at 20,000 x g. Pellets were resuspended in assay buffer and binding experiments were performed at 12°C for 20 min with glucagon (0.3 mg per ml) and dithiothreitol (5 mM) present. Binding is expressed in femtomoles per mg of protein as the difference between the amount of labeled angiotensin II bound in the absence and the presence of 5 x 10⁻⁶ M unlabeled angiotensin II. Organs were mixed from five animals. Liver and kidney subcellular fractions were also tested from individual animals. N.D., not determined.

| Organ       | Angiotensin II bound |
|-------------|----------------------|
|             | [3H]Angiotensin II    | [3H]Angiotensin II |
|             | (2 x 10⁻⁶ M)          | (2 x 10⁻⁶ M)      |
| Heart       | 0.04                 | N.D.              |
| Brain       | 0.1                  | N.D.              |
| Adrenal     | 6.8                  | 81.3              |
| Liver       | 0.4 (0.05-1.6)³      | 2.3 (0.2-9.0)³    |
| Kidney      | 0.3 (0.04-0.9)³      | 1.8 (0.1-3.6)³    |
| Colon       | 0.01                 | 1.5               |
| Lung        | 0.5                  | 0.90              |
| Aorta       | 0.5                  | 0.90              |

³ Range found in individual samples.

than angiotensin II binds preferentially and with high affinity to adrenal cortex subcellular particles.

Other Properties of Angiotensin II Receptors in Bovine Adrenal Cortex—It has been mentioned above that adrenal cortex subcellular particles demonstrated changes in their angiotensin II binding properties dependent on the time which elapsed after the adrenal glands were obtained. These changes could be enhanced by freezing and thawing and were independent from the purity of the receptor preparation (Fig. 13).

Whereas binding-inhibition studies with angiotensin analogues and fragments, and their respective affinities for the receptor, were reproducible in freshly prepared subcellular particles from bovine adrenal cortex, this was not always the case in frozen and thawed preparations.

CaCl₂ (2.5 and 5 mM) and ethylenediaminetetraacetic acid (sodium salt), 5 mM had no significant effect on the binding of angiotensin II to the adrenal cortex receptor preparation (Fig. 14). Trypsin pretreatment of subcellular particles leads to loss of angiotensin II binding, indicating that a protein component of the receptor is essential for specific angiotensin binding (Table V).

**DISCUSSION**

The angiotensin II binding sites of the adrenal cortex exhibit features consistent with those of a biologically relevant receptor, with high affinity, limited capacity, and high specificity for the hormonally active form of the trophic peptide. It is likely although yet unproven, that such sites represent the receptors responsible for the activation of aldosterone secretion by angiotensin II in vivo. The affinity of the particulate adrenal receptors for angiotensin II is comparable with that of the smooth muscle receptors (0.8 x 10⁵ M⁻¹) demonstrated in aortic tissue by Meyer and colleagues (10). In both cases, the magnitude of the association constant is lower than might be anticipated from the known plasma concentration of the circulating peptide (2 x 10⁻¹⁵ M), but this could be due to the complicating effect of peptide degradation upon steady state and kinetic binding studies or could reflect a requirement for local generation of angiotensin II in responsive target tissues. It is also possible that the observed affinity of binding sites studied in tissue ho-
Tensin II whether additions were present or not. Since the concentration of unlabeled peptide which leads to a \((B/B_0 \times 100)\)

Final assay volume was 0.12 ml; glucagon (0.3 mg per ml) and [\(\text{H}\)]-angiotensin II (final concentration, 0.5 nM) were added. (Final volume was 210 \(\mu\)l.) After 12 min samples were filtered as described under "Methods." Control values were obtained in samples to which L-1-tosylamido-2-phenylethyl-chloromethyl ketone-treated trypsin together with pancreatic trypsin inhibitor was added after the preincubation period. Each value is the mean ± S.D. of five determinations.

| Pretreatment conditions | [\(\text{H}\)]-Angiotensin bound |
|-------------------------|---------------------------------|
| Control                 | 41.3 ± 3.3                      |
| Trypsin for 10 min      | 11.3 ± 0.35                     |
| Trypsin and trypsin inhibitor for 10 min | 34.5 ± 1.33 |

mogenates are reduced by physical treatment and receptor lability to a value below that existing in the intact tissue. Whatever the mechanism, the receptor sites for angiotensin II in the adrenal cortex showed a significantly lower affinity for the peptide than those of the specific adrenal receptors for adrenocorticotropic hormone, which lie in the ranges of \(10^7\) and \(10^8 \, \text{M}^{-1}\) (27). The latter sites are known to be extremely labile, a factor which may also be of relevance to determination of the binding constants for angiotensin II in the adrenal cortex.

The most significant aspects of the present study are the constant finding of preferential binding of angiotensin II, rather than angiotensin I, by the particulate receptor fractions prepared from bovine adrenal cortex and rat adrenals, and the main location of angiotensin II binding sites in microsomal membrane vesicles rather than in mitochondria. Selective binding of angiotensin II has been demonstrated with \(\text{H}\)- and \(\text{H}\)-labeled peptides, and also by binding-inhibition studies with the unlabeled peptides. The reason for the difference between these observations and the earlier studies of Goodfriend and Lin (8, 11) is not apparent, but a possible explanation may lie in the occasional finding during these studies of a relative loss of specificity in the angiotensin binding sites of stored adrenal particles. A marked feature of the adrenal binding studies was the rapid degradation of angiotensin II during incubation of washed adrenal cortex particles with the labeled peptide, a process which appears to be relatively independent of receptor binding. Thus, peptide eluted from the binding sites at low pH showed substantial retention of binding activity and minimal evidence of degradation on thin layer chromatography. This was in contrast to unbound peptide remaining in the incubation medium, which showed marked loss of binding activity and extensive degradation to labeled peptide fragments.

### Table V

| Pretreatment conditions | [\(\text{H}\)]-Angiotensin bound |
|-------------------------|---------------------------------|
| Control                 | 41.3 ± 3.3                      |
| Trypsin for 10 min      | 11.3 ± 0.35                     |
| Trypsin and trypsin inhibitor for 10 min | 34.5 ± 1.33 |

Fig. 12. Displacement of \(\text{H}\)-angiotensin I and \(\text{H}\)-angiotensin II by angiotensins I and II, and the (Sar\(^3\), Ala\(^4\)) angiotensin II analogue from bovine adrenal cortex (A) and medulla (B) subcellular particles. Conditions were as given for Fig. 11. Labeled angiotensin was 0.03 nM. The final concentration of unlabeled peptides is given on the abscissa. Angio I and II, angiotensin I and II, respectively.

Fig. 13 (left). Effect of freezing and thawing on angiotensin II receptors (bovine adrenal cortex). The figure shows displacement curves for \(\text{H}\)-angiotensin II (final concentration, 1.4 nM) by unlabeled angiotensin II of a freshly prepared 1500 \(\times\) g particulate fraction (0.71 mg of protein) and Fraction 1 (0.36 mg of protein) after sucrose gradient centrifugation (see Table II), and of thawed fractions after storage in liquid nitrogen for 24 hours. Final assay volume was 0.12 ml; glucagon (0.3 mg per ml) and dithiothreitol (5 mM) were present (\(T = 22^\circ\)). In the absence of unlabeled angiotensin II, 1500 \(\times\) g particles bound 50 fmoles of \(\text{H}\)-angiotensin per sample and Fraction 1 bound 66 fmoles per sample. After storage and thawing, binding was reduced to 31 and 41 fmoles per sample, respectively.

Fig. 14 (right). Influence of calcium and ethylenediaminetetraacetic acid on angiotensin II receptors. Displacement curves for \(\text{H}\)-angiotensin II (final concentration, 0.5 nM) in the absence or presence of CaCl\(_2\) (2.5 and 5.0 mM) and 5 mM ethylenediamine tetraacetic acid (sodium salt) are shown. A 20,000 \(\times\) g particulate fraction from bovine adrenal cortex (0.25 mg of protein) in a final volume of 0.16 ml was tested. Twenty femtomoles of \(\text{H}\)-angiotensin II were bound per sample in the absence of unlabeled angiotensin II whether additions were present or not. Since the concentration of unlabeled peptide which leads to a \((B/B_0 \times 100)\)

value of 50 is a function of initial tracer concentration, affinity of the receptor and its concentration, neither the total receptor concentration nor its affinity seems to be influenced by either calcium (2.5 or 5.0 mM) or ethylenediamine tetraacetic acid, although reciprocal changes in affinity and receptor concentration cannot be excluded by this type of analysis.
In this respect, angiotensin II-receptor studies with bovine adrenal cortex subcellular particles posed similar difficulties as encountered with other hormones, e.g. insulin (28) or glucagon (29). Both hormones are rapidly inactivated by highly purified liver cell membranes, and the glucagon-inactivating process even copurifies with the glucagon receptor and the glucagon-activated adenylate cyclase (29).

Such rapid degradation of the ligand obviously could influence the kinetic and steady state data obtained during angiotensin-binding studies and may be an important factor in determining the magnitude of the affinity constants derived from angiotensin uptake data in muscle and adrenal homogenates. This problem is presently under investigation to clarify the mechanism of angiotensin II degradation in adrenal binding fractions and to optimize the analysis of binding data in systems which are susceptible to rapid decay of receptors and trophic hormones.

The relative potencies of angiotensin fragments and analogues in the adrenal receptor binding-inhibition system with labeled angiotensin II were closely related to their biological activities on vascular smooth muscle as determined by conventional bioassay procedures. This provided additional evidence for the biological relevance of the angiotensin receptors of the adrenal cortex. In particular, the extremely low binding-inhibition potency of the 3-8 heptapeptide of angiotensin II was consistent with the lack of effect of this peptide upon aldosterone secretion in vivo. This provided additional evidence for the biological relevance of the angiotensin receptors of the adrenal cortex. In particular, the extremely low binding-inhibition potency of the 3-8 heptapeptide of angiotensin II was consistent with the lack of effect of this peptide upon aldosterone secretion in vivo. This provided additional evidence for the biological relevance of the angiotensin receptors of the adrenal cortex.
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