Biochemical Pharmacology, Vol. 25, pp. 1031–1034. Pergamon Press, 1976. Printed in Great Britain.

ANGIOTENSIN II BINDING TO *ZONA GLOMERULOSA* CELLS FROM RABBIT ADRENAL GLANDS

STEVE GURCHINOFF, PHILIP A. KHAIRALLAH,* MARIE AUDE DEVYNCK and PHILIPPE MEYER

Research Division Cleveland Clinic Foundation, Cleveland, Ohio 44106, U.S.A.
and *Hopital Necker, INSERM U.7, 75015 Paris, France

(*Received* 14 *May* 1975; *accepted* 5 *September* 1975)

Abstract Tritiated angiotensin II binds in a highly specific manner to zona glomerulosa cells prepared from the adrenal cortex of male rabbits. The reaction is a time-dependent process which obeys second-order kinetics ($k_1 = 2.4 \times 10^5$ M^{-1} sec^{-1}) and reaches saturation in 5–7 min. Dissociation of the angiotensin II-cell complex is rapid ($t_{1/2} = 100$ sec) and obeys first-order kinetics for the first 3 min ($k_{-1} = 6.9 \times 10^{-3}$ sec^{-1}).

Increased binding was observed with NaCl concentrations from 0 to 40×10^{-3} M; however, concentrations from 40 to 140×10^{-3} M decreased binding. Neither $MgCl_2$ nor $CaCl_2$ at concentrations of 0 to 4×10^{-3} M alter the binding of angiotensin II to zona glomerulosa cells. A significant decrease in binding was observed with increasing concentrations of KCl (0 to 140×10^{-3} M).

Temperature studies indicate that initially binding is more rapid at 37° (37° > 25° > 0°). However, binding of angiotensin II decreases after 3 min at 37° and after 7 min at 25°. Binding at 0° did not reach a plateau in the 15-min period studied.

The pharmacologic action of the octapeptide angiotensin II has been extensively studied [1]. Some of the actions of the hormone are contraction of smooth muscle and stimulation of aldosterone production in the adrenal cortex. Recent investigations into these target tissues have demonstrated receptors for angiotensin II in intact rabbit aorta, [2], in microsomes from the intimal-medial layer of rabbit aorta [3], and in a membrane fraction from plasma membranes [4] of the same tissue. These investigators have also reported the solubilization of an angiotensin-binding component [5] from rabbit aorta plasma membranes.

Evidence for angiotensin II receptors has also been reported in bovine and rat adrenal subcellular particles [6] and in zona glomerulosa cells prepared from the cortex of rat adrenals [7]. Brecher and co-workers [8] have shown that tissue specificity to angiotensin II binding is higher in homogenized rat adrenal capsules than in decapsulated adrenals, liver, aorta, kidney or uterus which confirms studies done by Glossmann *et al.* [6]. Specific binding of ^{125}I-labeled angiotensin II to bovine and rat adrenal subcellular fractions was found to be influenced by the cation content of the incubation medium [9] and inhibited by guanyl nucleotides [10].

This study was undertaken to characterize the binding of angiotensin II to *Zona glomerulosa* (*z.g.*) cells prepared from the cortex of rabbit adrenals and to determine whether binding characteristics are similar in different species.

METHODS

Tritiated angiotensin II (^{3}H]AII, sp. act. 70 Ci/mmole) was a gift from the Centre d'Etudes Nucleaires de Saclay (France). The material was stored in small aliquots in liquid nitrogen. Hypertensin was purchased from Ciba–Geigy Corp. (Summit, New Jersey). Trypsin, trypsin inhibitor, deoxyribonuclease (DNAase) and bovine serum albumin (BSA) were purchased from Sigma Chemical Corp. (St. Louis, Missouri). Collagenase was obtained from Worthington Biochemical Corp. (Freehold, New Jersey).

Male rabbits of the New Zealand strain (2.5–4.0 kg) were given air emboli via the ear vein. A midline incision was made, the adrenals quickly removed and placed in cold buffer [0.02 M Tris–HCl (pH 7.4), and 0.12 M NaCl] containing 0.2% (w/v) glucose and 4% (w/v) BSA. Extraneous material was dissected from the adrenals, each gland was halved, and the medulla and inner cortical mass were gently scraped away. The capsular layer with adherent outer cortex was minced finely with scissors. *Zona glomerulosa* cells were prepared by the trypsin–collagenase digestion procedure[11] under an atmosphere of 95% O_2 and 5% CO_2. All subsequent cell suspensions and incubations were performed with plastic labware. Isolated cells were resuspended in 0.02 M Tris–HCl (pH 7.4) and 0.12 M NaCl containing 0.2% glucose and 0.1% BSA. An aliquot of the cell suspension was stained with an equal volume of 0.5% nigrosin or 0.2% trypan blue exclusion dyes and counted in a Neubauer hemocytometer. The yield of *Zona glomerulosa* cells was $1–1.5 \times 10^6$ cells per adrenal and contamination by *Zona fasiculata* was less than 5 per cent. Protein concentrations were determined by the method of Lowry *et al.* [12].

Incubations were performed at 25° unless stated otherwise. Labeled and unlabeled angiotensin II were added to the incubation medium in small volumes using microliter pipets. Cation studies were performed after resuspending pellets in the proper salt concentration. Aliquots (200 μl) of the incubation medium were placed on HAWP millipore filters (0.45 μm). The filters were washed with 10 ml of buffer, air-dried, and counted in a Packard liquid scintillation counter at 23 per cent efficiency.

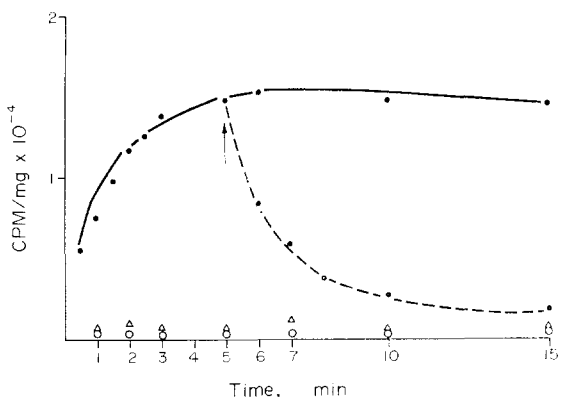

Fig. 1. Binding of 10^{-8} M [^{3}H] angiotensin II to *Zona glomerulosa* cells at 25°. The arrow indicates time at which 1000-fold excess unlabeled angiotensin was added to the incubation. The dotted line shows the dissociation of the complex. Open circles depict the binding with cells heated at 70° and open triangles the binding with 1000-fold excess unlabeled angiotensin.

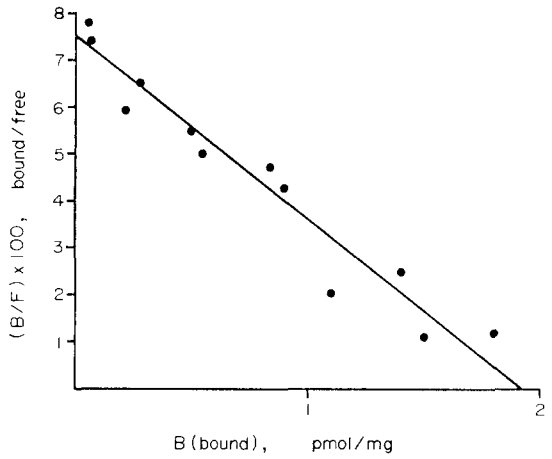

Fig. 2. A Scatchard plot of the binding of [^{3}H] angiotensin II to *Zona glomerulosa* cells. Each point is the average of triplicate determinations and has been corrected for non-specific binding. Incubation was at 25° for 5 min at concentrations of angiotensin II between 10^{-9} and 10^{-7} M.

RESULTS

The binding of 10^{-8} M [^{3}H] angiotensin II vs time is depicted in Fig. 1. Saturation of binding was reached between 5 and 7 min and the half-life of the dissociation (induced by addition of 1000-fold excess unlabeled angiotensin II after a 5-min incubation) of the complex was approximately 100 sec. Non-specific binding is very low as is the binding to cells which have been placed in a 70° water bath for 10 min.

The association of the angiotensin-cell complex follows second-order kinetics while the dissociation of the complex follows first-order kinetics (Table 1) for the first 3 min [5, 13]. The association rate constant at 25° is $k_1 = 2.4 \times 10^5$ M^{-1} sec^{-1} and the dissociation rate constant is $k_{-1} = 6.9 \times 10^{-3}$ sec^{-1}.

A Scatchard plot [14] of the specific binding of angiotensin II to *Zona glomerulosa* cells is presented in Fig. 2. A single high affinity binding site is characterized with [^{3}H] angiotensin II at concentrations between 10^{-9} and 10^{-7} M. The number of sites is approximately 2 pmole/mg protein and the apparent dissociation constant is 3.8×10^{-8} M. The dissociation constant at equilibrium (k_{-1}/k_1) is 2.9×10^{-8} M (Table 1).

Incubation of *Zona glomerulosa* cells with $1–2 \times 10^{-8}$ M [^{3}H] AII at 0°, 25°, and 37° vs time is shown in Fig. 3. Binding at 0° appears to increase during the 15-min incubation and is lower than the binding at 25° and 37°. At 25° binding reaches a maximum between 5–7 min then steadily decreases. Binding at 37° reaches a maximum in 3–5 min then decreases.

Table 1. Kinetic constants for the binding of angiotensin II to *Zona glomerulosa* cells

Association rate constant (k_1)	2.4×10^5 M^{-1} sec^{-1}
Dissociation rate constant (k_{-1})	6.9×10^{-3} sec^{-1}
Dissociation constant (K_d)	
From Scatchard plot	3.8×10^{-8} M
From k_{-1}/k_1	2.9×10^{-8} M

The effect of increasing NaCl and KCl concentrations on angiotensin binding is depicted in Table 2. Binding increases from 0 to 40 mM NaCl then begins to decline to below the zero value at 150 mM. Increasing concentrations of KCl decrease binding, with binding at all concentrations significantly different from the zero value. In order to be sure if the effect was due to increasing KCl concentrations or increasing ionic strength, another study was performed with a buffer containing 20 mM NaCl as opposed to the original buffer which contains 120 mM NaCl. Binding increases with the lower concentration of NaCl as compared to 120 mM NaCl, and then decreases as KCl concentration becomes greater than 20 mM.

Table 3 shows that increasing concentrations of $MgCl_2$ and $CaCl_2$ do not significantly alter the binding of angiotensin II to *Zona glomerulosa* cells.

DISCUSSION

Some of the criteria for binding studies, as set forth by Cuatrecasas [15], are: binding must demonstrate strict substrate specificity, saturability, reversibility, target-cell specificity and affinity, and kinetic rate constants that correlate with the biologic properties of the hormone.

The binding data in this study have met most of these criteria. Angiotensin II binding to *Zona glomerulosa* cells is specific (non-specific binding is less than 5 per cent), saturable and readily reversible, and kinetic constants correlate well with the biologic properties of the hormone. *Zona glomerulosa* cells responded to angiotensin II (1×10^{-8} to 5×10^{-7} M) with a dose dependent increase in aldosterone synthesis [16]. The concentration (10^{-8} M) of hormone used in this study also compares favorably with the amount necessary for contraction of aorta [4] and release of calcium from microsomes [3].

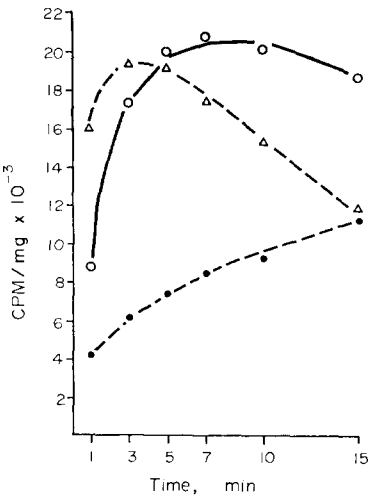

Fig. 3. Incubation of *Zona glomerulosa* cells with tritiated angiotensin II vs temperature. Closed circles (●) 0°, open circles (○) 25°, open triangles (△) 37°.

The kinetic data (Table 1) of angiotensin II binding to *Zona glomerulosa* cells from rabbit adrenals compare favorably to those for solubilized receptors from rabbit aorta ($k_1 = 2.5 \times 10^5$ mole^{-1} sec^{-1}; $k_{-1} = 4.1 \times 10^{-3}$ sec^{-1}; $k_{-1}/k_1 = 2.0 \times 10^{-8}$ M) [5] and to the association constant for bovine adrenal subcellular particles ($k_1 = 2.4 \times 10^5$ mole^{-1} sec^{-1}) but varies slightly from the dissociation and equilibrium constants ($k_{-1} = 5 \times 10^{-4}$ sec^{-1}; $k_{-1}/k_1 = 2 \times 10^{-9}$ M) [6] due to the long half-time of dissociation of the complex. These results indicate the binding of angiotensin II is similar for the three different systems especially since all three systems report at least one receptor site is being measured at 1–2 pmoles of binding sites per mg of protein (100,000 cells is approx 45 μg protein). Kinetic data for angiotensin II binding to rat adrenal preparations have not been reported, however, Glossmann and co-workers [6] indicate that angiotensin II affinity for rat adrenal subcellular particles is ten times greater than in bovine adrenal subcellular particles.

Binding studies of this report show no absolute requirement for cations. Angiotensin II binds to *Zona glomerulosa* cells without Na^+ in the buffer. The decrease in binding with increasing concentrations of K^+ may be due to an increase in ionic strength of the medium rather than to the presence of KCl. Neither calcium nor magnesium significantly affect the binding of angiotensin to *Zona glomerulosa* cells from rabbit adrenals (Table 3). Glossmann and co-workers [9] have shown that sodium and potassium ions increased binding of angiotensin II to bovine adrenal cortex receptors while rubidium, cesium, lithium, and magnesium did not, and in high concentrations caused inhibition of binding. Increasing amounts of Na^+ were found to enhance angiotensin II activity in rat pressor and uterotonic assay systems with Ca^{2+} twice as effective as Na^+ [17] Devynck and co-workers [18] have reported that sodium in the incubation medium did not alter the binding of angiotensin II to smooth muscle cell membranes but variations in binding were observed with increasing calcium concentrations. Addition of $CaCl_2$ (2.5 and 5 mM) had no significant effect on the binding of angiotensin II to bovine adrenal cortex receptor preparations [6]. A possible explanation for these differences may be that sodium and potassium do not affect intact cells but do affect the subcellular particles from bovine adrenals.

Binding studies performed at different temperatures indicate that angiotensin II binding to *Zona glomerulosa* cells decreases with time at 25° and 37° but increases at zero degrees (Fig. 3). Rapid degradation

Table 3. The effects of increasing $CaCl_2$ and $MgCl_2$ on angiotensin II binding on *Zona glomerulosa* cells for 5 min at 25°.

Concn (mM)	$CaCl_2$† c.p.m./mg protein‡	$MgCl_2$* c.p.m./mg protein‡
0	14035 ± 964	11163 ± 800
1	15038 ± 1311	10485 ± 769
1.5	14787 ± 977	10844 ± 730
2.0	14026 ± 1451	10988 ± 969
2.5	14644 ± 1866	10449 ± 447
3.0	14803 ± 1189	10682 ± 609
3.5	13302 ± 986	10898 ± 1168
4.0	13285 ± 1103	10954 ± 1015

Determinations were carried out in 0.02 M Tris–HCl (pH 7.4), 0.12 M NaCl, 0.2% (w/v) glucose, and 0.1% BSA.

* Values not significantly different from zero value.

† Concentration of [^{3}H] Angiotensin II was 2×10^{-8} M.

‡ Values are mean ± S.D.

Table 2. The effects of increasing NaCl and KCl concentrations on the binding of angiotensin II to *Zona glomerulosa* cells for 5 min at 25°

Concn. (mM)	NaCl c.p.m./mg protein†	KCl* c.p.m./mg protein†	KCl‡ c.p.m./mg protein†
0	9232 ± 819	7798 ± 1006	9648 ± 767
20	10,679 ± 1062 P < 0.01	6734 ± 798 P < 0.025	9724 ± 1741 N.S.
40	11,492 ± 777 P < 0.001	6109 ± 895 P < 0.005	9086 ± 1179 N.S.
80	10,924 ± 784 P < 0.001	5302 ± 558 P < 0.001	7215 ± 423 P < 0.001
100	10,746 ± 741 P < 0.001	4839 ± 754 P < 0.001	6898 ± 425 P < 0.001
120	9550 ± 1002 N.S.	4454 ± 543 P < 0.001	6588 ± 394 P < 0.001
140	7875 ± 1245 P < 0.025	3481 ± 716 P < 0.001	5726 ± 343 P < 0.001

* Incubation medium contained 120 mM NaCl.

† Values are means ± S.D.

‡ Incubation medium contained 20 mM NaCl.

N.S. means not statistically significant.

of angiotensin II during incubation has been reported by Glossmann *et al.* [6] at 22° and 37°. These authors have shown that the peptide eluted from the binding sites had substantial retention of binding activity and minimal degradation. This was found to be in marked contrast to unbound peptide in the incubation medium which had extensive degradation and loss of binding activity.

Studies of possible reasons for decreased binding of angiotensin have not been completed. Binding studies with angiotensin analogs and fragments may lead to a better understanding of this phenomenon. The 2–8 heptapeptide was almost equipotent with the octapeptide in binding inhibition and similar in stimulating aldosterone secretion [19]. Chiu and Peach [16] suggest that des-Asp^1-angiotensin II has a higher affinity than does angiotensin for the angiotensin receptor in the adrenal cortex and the steroidogenic response of the two peptides is similar. The 2–8 heptapeptide has been reported to be nearly equipotent to the octapeptide in binding inhibition studies on bovine adrenal cortex particulate fractions [6]. Bravo and co-workers [20] report that [1-des (Aspartic acid), 8-Isoleucine] angiotensin II is a specific antagonist of the steroidogenic effect of angiotensin II but does not affect its pressor action. These findings all seem to suggest that fragments of angiotensin play an important role in the adrenal cortex.

Since there is little circulating heptapeptide in the blood, Chiu and Peach [16] have suggested that angiotensin II may stimulate synthesis of aldosterone by production of heptapeptide by aminopeptidases. If this postulation is true then perhaps the angiotensin receptor site on *Zona glomerulosa* cells is an enzyme which converts the octapeptide to the heptapeptide inducing the steroidogenic response.

Further studies on angiotensin II binding to *Zona glomerulosa* cells prepared from rabbit adrenals will concern the effects of various angiotensin II analogs and fragments; to determine why binding with increasing temperatures appears to decrease with time; and an attempt at solubilizing receptors and isolating membrane fragments that may be involved with angiotensin binding.

Acknowledgements This research was supported in part from National Institutes of Health grant HL-6835, N.I.H. Training Grant HL-5126, and partial support from The American Heart Association Northeast Ohio Affiliate, Inc.

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