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Specific receptors for des-Asp[1]-angiotensin II ("angiotensin III") in rat adrenals

(aldosterone)

MARIE-AUDE DEVYNCK*, MARIE-GABRIELLE PERNOLLET*, P. GEOFFREY MATTHEWS*, MAHESH C. KHOSLA†, F. MERLIN BUMPUS†, AND PHILIPPE MEYER*

* Physiologie et Pharmacologie Cardiovasculaire et Rénale, Institut National de la Sante et de la Recherche Médicale, U.7, Hôpital Necker, 75015 Paris, France; and † Research Division, Cleveland Clinic Foundation, Cleveland, Ohio 44106

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ABSTRACT **The specific binding of angiotensin II and des-Asp[1]-angiotensin II ("angiotensin III") to rat adrenals was studied with the use of the tritiated peptides. The binding sites having maximal affinity for angiotensin II were characterized by an equilibrium dissociation constant of 3.3 to 5.2 × 10^{-9} M. Angiotensin III was able to interact with these sites, and also with a class of sites with very high affinity, characterized by an equilibrium dissociation constant of 1 to 2 × 10^{-10} M. These sites exhibited a greater affinity for the heptapeptide angiotensin III than for the octapeptide angiotensin II. These findings, together with the known potent aldosterone stimulating effect of angiotensin III and its presence in rat plasma, suggest that this heptapeptide could be the physiologically important steroidogenic angiotensin in this species.**

Angiotensin II-(2-8)-heptapeptide (des-Asp[1]-angiotensin II or "angiotensin III") is a naturally occurring product of the renin-angiotensin system. It has been believed that angiotensin III may be derived from angiotensin II by the action of aminopeptidase A (1). Recent series have proposed that angiotensin III could also originate from the action of converting enzyme on the nonapeptide, des-Asp[1]-angiotensin I (2, 3). *In vivo* (4–6) and *in vitro* (7, 8) studies have indicated that angiotensin III is at least as potent as angiotensin II in stimulating aldosterone biosynthesis. In addition, studies performed with competitive inhibitors, obtained by substitution of the COOH-terminal Phe[8] of angiotensin II and III with aliphatic amino acids (e.g., Ile), have suggested that angiotensin III could be the compound ultimately responsible for angiotensin-mediated aldosterone production. [Sar[1], Ala[8]]angiotensin II and [Sar[1], Ile[8]]angiotensin II, which markedly inhibit the pressor effect of angiotensin II, have less antagonistic action on the steroidogenic effect of the hormone (9, 10); conversely, the angiotensin III antagonist, des-Asp[1]-[Ile[8]]angiotensin II, is a potent inhibitor of the steroidogenic action of both angiotensin III and angiotensin II (11–13). These studies, using 8-substituted derivatives as probes of optimal structure-activity relationships, suggest that the octapeptide is required at the level of vascular receptors, whereas the COOH-terminal heptapeptide could be the active structure in the adrenal cortex. Our previous studies (14, 15) on hormone–receptor interaction demonstrated that the presence of a chain-length of eight amino acid residues of angiotensin II is indeed necessary for an optimal binding to smooth muscle receptors. However, [Sar[1]]angiotensin II, which is not readily degraded by plasma aminopeptidases, has been reported to have superior steroidogenic potency than angiotensin II or angiotensin III; thus, the necessity for biological transformation of the octapeptide angiotensin II into the heptapeptide angiotensin III in the adrenals has been questioned. The present study was undertaken to seek an unequivocal answer to this question by demonstrating the presence of specific receptors for angiotensin III in the rat adrenals.

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MATERIALS AND METHODS

Angiotensin II ([Asp[1], Ile[5]]angiotensin II) and angiotensin III (des-Asp[1]-[Ile[5]]angiotensin II) were tritiated by catalytic substitution of iodinated peptides (16) and their purity was assessed by thin-layer chromatography. The specific activity of angiotensin II was 45.6 Ci/mmol. Two batches of [^{3}H]angiotensin III of 22.3 and 30 Ci/mmol were used. Peptide analogues were synthesized at the Research Division of the Cleveland Clinic, Cleveland, OH. Experiments were performed on particulate fractions of adrenal tissue prepared from Wistar rats by differential centrifugation between 3000 and 30,000 × *g* (17). The binding of tritiated angiotensin to adrenal particulate fractions was studied within 4 hr of their preparation in a volume of 500 μl or 1 ml of incubation medium (100 mM KCl/5 mM $MgCl_2$/10 mM histidine at pH 6.8). Careful attention was paid to the quantity of protein per sample in order to fulfil the criterion for precise determination of hormone–receptor interactions of high affinity (18). Incubations were performed in the presence of 80–150 μg of protein at 29°. Membrane-bound and free radioactivity were separated on Millipore filters HAWP 0.45 μm. Specifically protein-bound radioactivity was considered as the difference between that bound in the absence and that bound in the presence of unlabeled peptide. All estimations were preformed in triplicate, and membrane-bound radioactivity was counted for 50 min to ensure more precision when tritiated peptides were used at low concentrations. As an example, under usual conditions with [^{3}H]angiotensin III, 7 × 10^{-11} M, total bound radioactivity was of the order of 2000 counts; nonspecifically bound radioactivity was 300–400 counts. The concentration of protein in the incubation medium was determined by the method of Lowry *et al.* (19).

RESULTS AND DISCUSSION

The time-courses of [^{3}H]angiotensin II and [^{3}H]angiotensin III binding to the rat adrenal particulate fraction were studied at 29° using initial concentrations ranging from 3 × 10^{-11} M to 5 × 10^{-9} M. Specific binding of [^{3}H]angiotensin II and [^{3}H]-angiotensin III reached equilibrium at 30 and 45 min, respectively, for the lowest concentrations. Equilibrium conditions were maintained for at least 60 min. This suggested no marked inactivation of ligands or loss of binding sites, confirming our earlier observations (17). Dissociation kinetics of [^{3}H]angiotensin II and [^{3}H]angiotensin III were studied by the addition of unlabeled ligands in excess to membranes equilibrated with tritiated peptides. Kinetic constant, calculated assuming a second

Table 1. Kinetic and equilibrium binding parameters of [^{3}H]angiotensins and their high affinity binding sites

Parameter	[^{3}H]Angiotensin III: High affinity sites	[^{3}H]Angiotensin III: Low affinity sites	[^{3}H]Angiotensin II*
Association rate constant (10^5 M^{-1} sec^{-1})			
From association studies	75 (4)†	—	6.6 (6)
Dissociation rate constant (10^{-4} sec^{-1})			
From association studies	7.6 (4)	—	22 (6)
From dissociation studies	4.0 (1)	—	28 (3)
Equilibrium dissociation constant (10^{-10} M)			
From k_{-1}/k_1	0.5–1.0	—	35 (26)
From Scatchard plots	1.5 (6)	46 (6)	52 (26)

* From ref. 17.

† Parentheses: number of determinations from which the value given has been obtained.

order rate reaction for association and a pseudo-first order rate reaction for dissociation, are indicated in Table 1.

The concentration dependence of angiotensin binding was studied over initial peptide concentrations ranging from 3×10^{-11} to 1×10^{-8} M. Scatchard plots (20) of data from binding studies performed at equilibrium with [^{3}H]angiotensin III and [^{3}H]angiotensin II are shown in Fig. 1. Between these concentrations angiotensin III binds to two classes of binding sites. The higher affinity binding sites were characterized by an apparent k_d of 1.5×10^{-10} M and a binding capacity of 1.2×10^{-13} mol/mg of protein. The lower affinity binding sites had an apparent k_d of 3.6×10^{-9} M and a binding capacity of 8.4×10^{-13} mol/mg. On the other hand, [^{3}H]angiotensin II at concentrations ranging from 3×10^{-11} to 1×10^{-8} M bound to a single class of sites (k_d at 29°, 3.5×10^{-9} M; binding capacity, 8.2×10^{-13} mol/mg). At concentrations higher than 2×10^{-8} M another class of binding sites of low affinity was observed (Fig. 1 *inset*). In spite of careful investigation at low concentrations of ligand it was not possible to identify angiotensin II binding sites of very high affinity.

The similarity of the binding characteristics of [^{3}H]angiotensin II and [^{3}H]angiotensin III to its low affinity sites suggested that these two ligands may bind to the same receptor. To test this possibility, the influence of angiotensin II on [^{3}H]angiotensin III binding was examined in a concentration dependence study of [^{3}H]angiotensin III binding performed in the presence and absence of a 20-fold concentration of unlabeled angiotensin II. Scatchard analysis of data showed a total suppression of the [^{3}H]angiotensin III sites of lower affinity but a failure of angiotensin II to suppress binding to the high affinity sites of angiotensin III.

Specificity of the binding of [^{3}H]angiotensin was studied by the binding inhibition induced by increasing concentrations of unlabeled angiotensin derivatives, a reflection of their respective affinities for the binding sites. To investigate the angiotensin III high affinity sites with only minimal influence from the other class, we limited the concentrations of competing ligands. The potencies of angiotensin derivatives competing for the [^{3}H]angiotensin sites are shown in Fig. 2. For the [^{3}H]-angiotensin III high affinity sites the order of potency was:

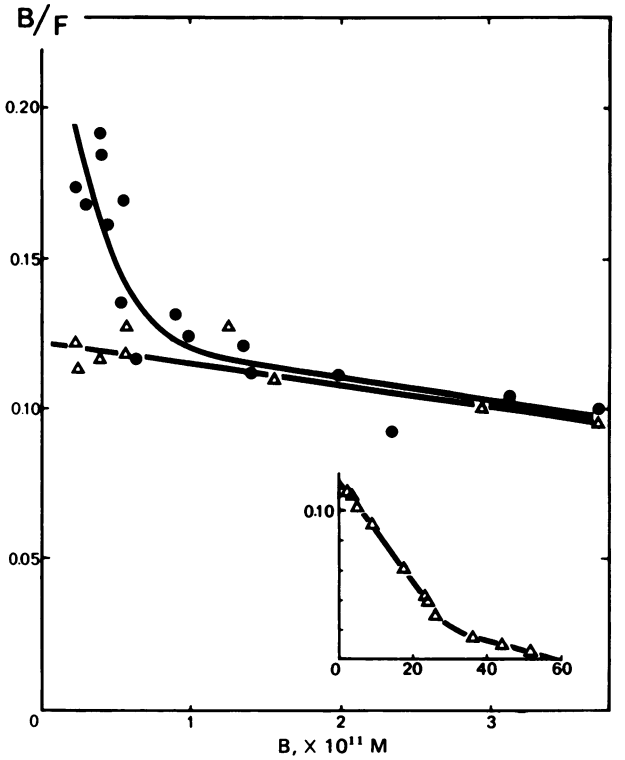

FIG. 1. Scatchard plots of binding data of (●) [^{3}H]angiotensin III and (△) [^{3}H]angiotensin II to rat adrenal particulate fraction derived from five separate experiments where binding of both peptides to the same prepared fraction was studied. Concentrations of tritiated angiotensins ranged from 5×10^{-11} to 1×10^{-9} M. [^{3}H]Angiotensin III demonstrated a curvilinear plot, while [^{3}H]angiotensin II showed a single class of binding sites. The slopes of the linear regression of data in the low concentration region for binding of [^{3}H]angiotensin III and that for [^{3}H]angiotensin II binding were significantly different ($P < 0.05$). (*Inset*) Similar examination of [^{3}H]angiotensin II binding across a greater range of ligand concentrations (1×10^{-10} to 5×10^{-7} M). [^{3}H]Angiotensin II bound to two saturable classes of sites with k_d values of 2.8×10^{-9} M and 2.5×10^{-8} M. B, bound; F, free.

angiotensin III > des-Asp^1-[Ile^8]angiotensin II > [Sar^1, Ile^8] angiotensin II > angiotensin II (Fig. 2*A*); for the [^{3}H]angiotensin II sites: angiotensin II > [Sar^1, Ile^8]angiotensin II > des-Asp^1-[Ile^8]angiotensin II > angiotensin III (Fig. 2*B*). The potent inhibitory effect of [Sar^1, Ile^8]angiotensin II for the angiotensin II sites is in agreement with our observations of an apparent k_d value of 5.1×10^{-9} M for binding on these membranes (measured directly with the tritiated analog), a value extremely close to that of angiotensin II (21). The binding inhibition induced by angiotensin II or angiotensin III on the low affinity [^{3}H]-angiotensin III sites was similar to that on the [^{3}H]angiotensin II sites (Fig. 2*C*), indicating that specificity was independent of the radioactive angiotensin used.

Scatchard plots demonstrating two classes of binding sites, as seen in the preparation with angiotensin III, have sometimes been considered to represent negative cooperativity (22). Simple negative cooperativity is an unlikely interpretation of the present study because of the marked change in specificity.

The present data have demonstrated the existence of two distinct receptor sites in this rat adrenal particulate fraction, one for angiotensin III, the other for angiotensin II. The distinction is substantiated by prominent differences in the affinities and number of binding sites of each group of sites, as well as the different specificities. The former exhibit affinity for a specific structure resulting from the removal of the

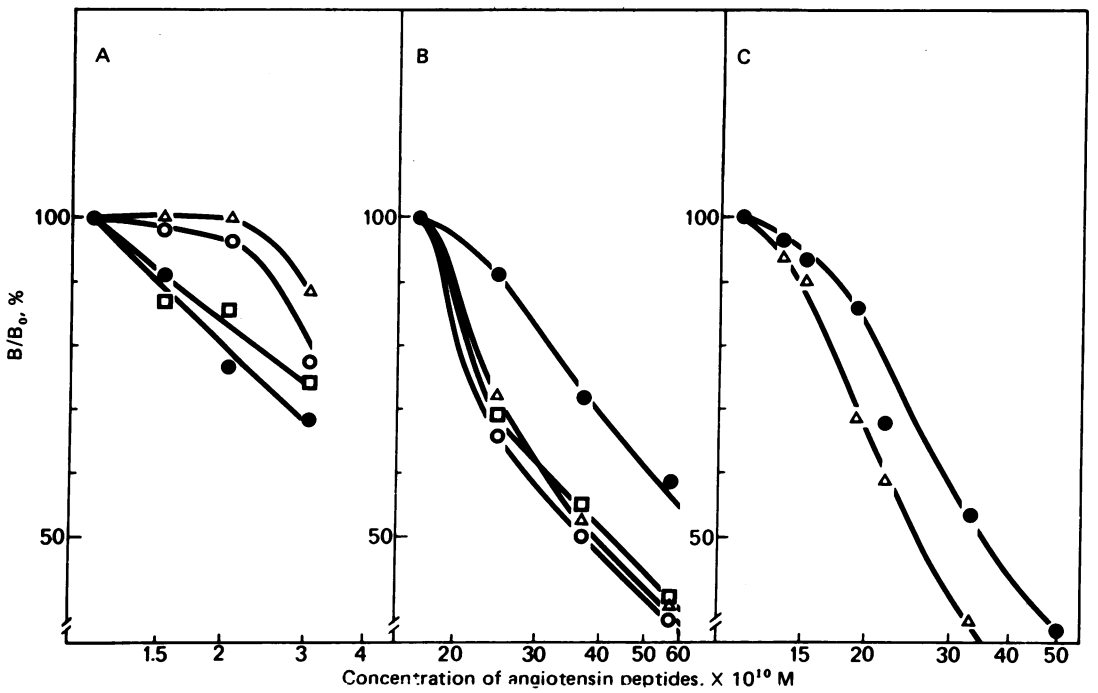

FIG. 2. Specificity of [^{3}H]angiotensin III and [^{3}H]angiotensin II binding to rat adrenals. ●, Angiotensin III; △, angiotensin II; □, des-Asp^1-[Ile^8]angiotensin II; ○, [Sar^1, Ile^8]angiotensin II. (*A*) High affinity sites. Adrenal particulate fraction protein was incubated with 1.0×10^{-10} M [^{3}H]angiotensin III in the presence of 0, 0.5, 1.0, and 2.0×10^{-10} M unlabeled angiotensin peptides. The angiotensin III binding sites showed greater affinity for the heptapeptide structure than for the octapeptide configuration. (*B*) Similar procedure to *A* using 1.7×10^{-9} M [^{3}H]angiotensin II and 0, 1, 2, and 4×10^{-9} M unlabeled peptides. The affinities of the octapeptides and des-Asp^1-[Ile^8]angiotensin II were approximately the same, whereas angiotensin III showed a lower affinity for this receptor. (*C*) Study of [^{3}H]angiotensin III low affinity sites using 1.2×10^{-9} M [^{3}H]angiotensin III. Better inhibition of binding was obtained with angiotensin II competition than with angiotensin III.

NH_2-terminal aspartic acid residue of the angiotensin octapeptide. The latter are essentially stereocomplements for the COOH-terminal end of the peptide, as shown previously (17), and are thus able to interact with both the octapeptide and the 2-8 heptapeptide fragment of angiotensin II. The efficiency of des-Asp^1-[Ile^8]angiotensin II in displacing both labeled ligands well could be related to a conformation with high flexibility. It agrees with its pharmacologically potent antagonism of angiotensin III- and angiotensin II-mediated aldosterone biosynthesis (11–13). The suppression of the low affinity group of sites by the octapeptide, leaving evidence only of the high affinity angiotensin III sites, further supports the independence of the two groups of sites. The identity of the angiotensin III low affinity sites and the angiotensin II sites is supported by their similarity in binding capacity and the strict specificity (higher affinity) in favor of the octapeptide structure. However, there is an apparent contradiction in that the similarity of k_d values does not fit with the preferential affinity for the octapeptide structure observed in binding inhibition studies. This is explained by the contribution of the high affinity [^{3}H]angiotensin III binding sites which leads to over-estimation of the affinity of [^{3}H]angiotensin III for its second class of sites.

The demonstration of angiotensin III binding sites in a particulate fraction enriched in plasma membranes (17) suggests that they may be located in this subcellular fraction in a similar fashion to the binding sites for angiotensin II in various target organs (15, 17, 23, 24). This does not exclude, however, the possibility of intracellular binding structures, as proposed previously (1, 25).

Plasma concentrations of angiotensin II and angiotensin III in the rat have been measured by radioimmunoassay of separated peptides (26). The concentrations were found to range between 10^{-11} and 10^{-10} M, the concentration of angiotensin III being twice that of angiotensin II. From the [^{3}H]angiotensin II k_d values of the present investigation, one may predict that at this plasma concentration the degree of occupancy of the angiotensin II receptors must be very low. On the other hand, angiotensin III can fully react with its high affinity receptor sites. This indicates that angiotensin-induced aldosterone secretion in the rat is probably regulated by circulating angiotensin III via its specific high affinity receptor. Pharmacological observations using angiotensin competitors have suggested that angiotensin III is an active component of the renin-angiotensin system in the adrenal of several species (4–6). Its biological role will be better defined by the wider identification of angiotensin III receptors and the study of the local concentration of ligands.

This study, demonstrating specific receptors for angiotensin III distinct from those of angiotensin II, raises the question of the significance of the angiotensin II binding sites. They may represent a complementary pathway for stimulation of aldosterone production in times of excessive stimulation of the renin-angiotensin system or, alternatively, they could be the receptors for angiotensin II produced locally by the adrenal isorenin-angiotensin system (27). The coexistence of receptors for angiotensin II and angiotensin III in the adrenal could explain the variability in responses seen with angiotensins II and III under different experimental conditions.

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1. Ledingham, J. G. & Leary, W. P. (1974) "Angiotensin," in *Handbook of Experimental Pharmacology*, eds. Page, I. H. & Bumpus, F. M. (Springer Verlag, New York), Vol. 37, pp. 111–125.
2. Campbell, W. B., Schmitz, J. M. & Itskovitz, H. D. (1976) *Endocrinology* **100**, 46–51.
3. Larner, A., Vaughan, E. D., Tsai, B. S. & Peach, M. J. (1976) *Proc. Soc. Exp. Biol. Med.* **152**, 631–634.
4. Blair-West, J. R., Coghlan, J. P., Denton, D. A., Funder, J. W., Scoggins, B. A. & Wright, R. D. (1971) *J. Clin. Endocrinol. Metab.* **32**, 575–578.
5. Lohmeier, T. E., Davis, J. O. & Freeman, R. H. (1975) *Proc. Soc. Exp. Biol. Med.* **149**, 515–518.
6. Campbell, W. B. & Pettinger, W. A. (1976) *J. Pharmacol. Exp. Ther.* **198**, 450–456.
7. Peach, M. J. & Chiu, A. T. (1974) *Circ. Res. Suppl. I* **34 & 35**, I-7–I-13.
8. Fredlund, P., Saltman, S. & Catt, K. J. (1975) *J. Clin. Endocrinol. Metab.* **40**, 746–749.
9. Spielman, W. S. & Davis, J. O. (1974) *Circ. Res.* **35**, 615–624.
10. Bravo, E. L., Khosla, M. C. & Bumpus, F. M. (1975) *Am. J. Physiol.* **228**, 110–114.
11. Sarstedt, C. A., Vaughan, E. D. & Peach, M. J. (1975) *Circ. Res.* **37**, 350–358.
12. Bumpus, F. M. & Khosla, M. C. (1975) *Clin. Sci. Mol. Med.* **48**, 15s–18s.
13. Peach, M. J., Sarstedt, C. A. & Vaughan, E. D. (1976) *Circ. Res. Suppl. II* **38**, II-117–II-121.
14. Devynck, M. A., Rouzaire-Dubois, B., Chevillotte, E. & Meyer, P. (1976) *Eur. J. Pharmacol.* **40**, 27–37.
15. Devynck, M. A. & Meyer, P. (1976) *Am. J. Med.* **61**, 758–767.
16. Morgat, J. L., Hung, L. T. & Fromageot, P. (1970) *Biochim. Biophys. Acta* **207**, 374–376.
17. Pernollet, M. G., Devynck, M. A., Matthews, P. G. & Meyer, P. (1977) *Eur. J. Pharmacol.*, **43**, 361–372.
18. Chang, K. J., Jacobs, S. & Cuatrecasas, P. (1975) *Biochim. Biophys. Acta* **406**, 294–303.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
20. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672.
21. Devynck, M. A., Koreve, V., Matthews, P. G., Meyer, P. & Pernollet, M. G. (1977) *Advances in Nephrology* (Year Book Medical Publishers Inc., Chicago) Vol. 7, in press.
22. De Meyts, P., Roth, J., Neville, D. M., Gavin, J. R. & Lesniak, M. A. (1973) *Biochem. Biophys. Res. Commun.* **55**, 154–161.
23. Glossmann, H., Baukal, A. J. & Catt, K. J. (1974) *J. Biol. Chem.* **249**, 825–834.
24. Forget, G. & Heisler, S. (1976) *Can. J. Physiol. Pharmacol.* **54**, 698–707.
25. Robertson, A. L. & Khairallah, P. A. (1971) *Science* **172**, 1138–1139.
26. Semple, P. F. & Morton, J. J. (1976) *Circ. Res. Suppl. II* **38**, II-122–II-126.
27. Ganten, D., Schelling, P., Vecsei, P. & Ganten, U. (1976) *Am. J. Med.* **60**, 760–772.