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ACCELERATED COMMUNICATION

Two Distinct Angiotensin II Receptor Binding Sites in Rat Adrenal Revealed by New Selective Nonpeptide Ligands

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SUMMARY

The nonpeptide angiotensin II antagonists Dup-89 and WL-19 displaced specific ^{125}I-angiotensin II binding in rat whole adrenal in a clearly biphasic manner, indicating the presence of high (nanomolar) and low (micromolar) affinity sites, each representing approximately 50% of the total maximal number of binding sites. Displacement studies using sufficient concentrations of either antagonist to prevent binding to its respective high affinity site revealed that the high affinity binding sites for Dup-89 and WL-19 were distinct and corresponded to the low affinity site of the other. Both binding sites were also present in the adrenal capsule (cortex) and adrenal decapsulated (medulla) tissue. The two ^{125}I-angiotensin II binding sites were also differentiated by their sensitivity to dithiothreitol and the relative affinities of angiotensin II, angiotensin III, and their respective Sar^1,Ile^8- and Ile^7-substituted antagonist analogs. Only Dup-89 (K_B = 13 nM) was effective in antagonizing angiotensin II-stimulated aldosterone release from dispersed adrenal capsular cells, indicating that this functional response is mediated by an action upon the ^{125}I-angiotensin II binding site at which Dup-89 has high affinity. Collectively, the data provide additional strong support for the presence of two distinct angiotensin II receptor subtypes in the rat adrenal.

The potential for multiple angiotensin II receptor subtypes in peripheral tissues and the central nervous system has been recognized for some time (1, 2). The data supporting these proposals were largely based upon the relative affinities or potencies of agonist and antagonist analogs of angiotensin in various target tissues and organs (1–5).

In the adrenal cortex, pharmacological evidence has indicated that angiotensin II and des-Asp^1-angiotensin II (angiotensin III) may mediate the release of aldosterone by activation of two distinct subtypes of angiotensin receptors. This view is based upon reports that angiotensin III is more potent on a molar basis than angiotensin II in stimulating steroidogenesis even though angiotensin II is more potent than angiotensin III in producing increases in blood pressure (6, 7). Moreover, the aldosterone-releasing action of angiotensin III is more effectively antagonized by 7-substituted antagonist analogs of angiotensin III than 8-substituted antagonist analogs of angiotensin II (6, 8). However, other investigators have provided evidence to the contrary and suggest that angiotensin II and angiotensin III act upon the same receptors in stimulating steroidogenisis (9).

Definitive evidence from radioligand binding studies for the existence of distinct angiotensin receptors in the rat adrenal is lacking. Studies with radiolabeled angiotensin II or angiotensin III have demonstrated the presence of high and low affinity sites in the rat adrenal (10, 11). However, such differences in affinity of agonists can be explained not only on the basis of two distinct binding sites but also by negative cooperativity of binding to a single site or by different guanine nucleotide-coupled states of the same receptor. In the present studies, two recently described nonpeptide angiotensin II antagonists (12, 13) (Fig. 1) are shown to have markedly different affinities for two subtypes of angiotensin II binding sites in rat adrenal. The data provide additional strong support for previous proposals for the existence of distinct angiotensin II receptors in this and other tissues (1–5).

Materials and Methods

Radioligand

^{125}I-angiotensin II was purchased from Dupont New England Nuclear.

^{125}I-Angiotensin II Binding Assay

Preparation of rat adrenal membranes. Rat whole adrenals or adrenals dissected into capsular and decapsulated layers were dissected free from fatty tissue. Membranes were prepared using the methods described previously for bovine adrenal cortical membranes (14). Briefly, adrenal tissues were homogenized in 50–100 volumes of 50 mM Tris·HCl (pH 7.4). The homogenates were centrifuged at 50,000 × *g* for 10 min. The resulting pellets were then washed twice in 100 mM NaCl, 5 mM EDTA, 10 mM Na_2HPO_4, 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4), by resuspension and centrifugation.

Binding assays. The membrane pellets were resuspended in 1000 ml of binding assay buffer (100 mM NaCl, 5 mM EDTA, 10 mM Na_2HPO_4, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml

ABBREVIATIONS: BSA, bovine serum albumin; DTT, dithiothreitol.

soybean trypsin inhibitor, 0.019 mg/ml *O*-phenanthroline, 2 mg/ml heat-denatured BSA, and 0.14 mg/ml bacitracin) for each gram of original tissue wet weight. In ^{125}I-angiotensin II binding assays, 10 μl of buffer (for total binding) or angiotensin II (1 μM final concentration, for nonspecific binding) and 10 μl of ^{125}I-angiotensin II (23–46 pM) were added to triplicate tubes. Receptor membranes (500 μl) were added to each tube to initiate the binding reaction. The reaction mixtures were incubated at 37° for 60 min. The reaction was terminated by filtration under reduced pressure through glass fiber GF/B filters, washed immediately four times with 4 ml of ice-cold Tris·HCl (50 mM, pH 7.4) containing 0.15 M NaCl. The radioactivity trapped on the filters was counted using a γ-counter.

Angiotensin II-Induced Aldosterone Release in Rat Dispersed Adrenal Cells

The method for the preparation of rat dispersed adrenal capsular cells was according to published procedures (15). Adrenals from 16–20 Sprague-Dawley rats were removed, placed in ice-cold Medium 199 or Krebs' solution without potassium, and bubbled with 95% O_2/5% CO_2. After the fatty tissues were removed, capsular tissues were obtained by gently pressing on the tissues to squeeze out the inner material. The combined capsular tissues were minced with a small scissors and washed twice by centrifugation at 120 × *g* for 5 min. The washed tissues were then incubated at 37° in 10 ml of digestion solution (Medium 199 without K^+, 1 mg/ml collagenase, 25 μg/ml DNase, 4% BSA, and 0.25 mg/ml soybean trypsin inhibitor) and bubbled with 95% O_2/5% CO_2. Tissues were pipetted through a 10-ml syringe 10 times to disperse the tissues. Dispersed tissues were filtered through a nylon mesh. The filtrate was saved and the undispersed tissues were further digested twice as described above.

The filtered dispersed cells were diluted with Medium 199 or Krebs' solution, without K^+ and containing 1% BSA, to a volume of approximately 35 ml and centrifuged at 100 × *g* for 15 min. Pellets were washed twice by resuspension and centrifugation. The combined cells from these harvests were resuspended in 25–40 ml of Medium 199 containing 0.14 mg/ml bacitracin, 0.2 mg/ml soybean trypsin inhibitor, 50 units/ml penicillin, and 50 μg/ml streptomycin.

For aldosterone release, 0.5 ml of cell suspensions was added to triplicate tubes containing 10 μl of buffer (basal release) or angiotensin II (10^{-11} to 10^{-6} M), with or without the test compounds (Dup-89 or WL-19) at the indicated concentration. The cells were incubated at 37° for 2 hr under 95% O_2/5% CO_2 atmosphere and then centrifuged at 1000 × *g* for 15 min. Supernatants were carefully removed and aliquots (100 μl) were used for radioimmunoassay of aldosterone, using a kit from Radioassay Systems Laboratories (Carson, CA).

Results

Effects of Dup-89 and WL-19 on ^{125}I-angiotensin II binding in whole rat adrenal. Specific ^{125}I-angiotensin II binding in rat adrenal was displaced by Dup-89 or WL-19 in a biphasic manner, with the high affinity sites amounting to approximately 50% of the total binding with each compound (Fig. 2). In contrast, the displacement produced by angiotensin II and angiotensin III (Fig. 2), and also by Sar^1,Ile^8-angiotensin II and Ile^7-angiotensin III (data not shown), was monophasic. In the presence of WL-19, at a concentration (0.3 μM) sufficient to occupy mainly its high affinity site, Dup-89 inhibited the remaining specific ^{125}I-angiotensin II binding in a monophasic manner, with an IC_{50} of 24 ± 7.8 nM (Fig. 3). Similarly, in the presence of Dup-89 (1 μM), at a concentration sufficient to occupy its high affinity site, WL-19 also inhibited specific ^{125}I-angiotensin II binding in a monophasic manner, with an IC_{50} value of 19 ± 9.1 nM (Fig. 3). These results indicated that the high affinity site of WL-19 corresponds to the low affinity site for Dup-89 and the high affinity site of Dup-89 corresponds to the site having a low affinity for WL-19. For the purpose of this report, we have designated the ^{125}I-angiotensin II binding

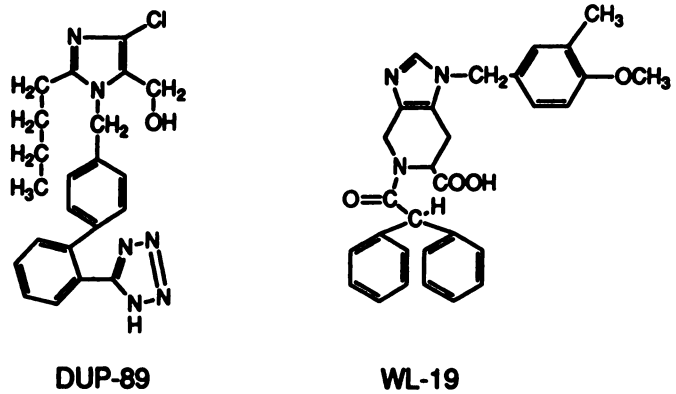

Fig. 1. Structure of Dup-89 and WL-19.

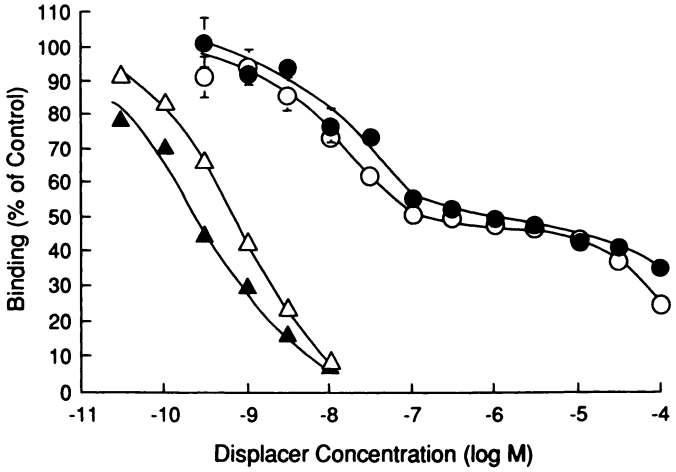

Fig. 2. Displacement of specific ^{125}I-angiotensin II binding in rat whole adrenal by Dup-89 (●), WL-19 (○), angiotensin II (△), and angiotensin III (▲). Data points represent the means ± standard errors of three independent experiments.

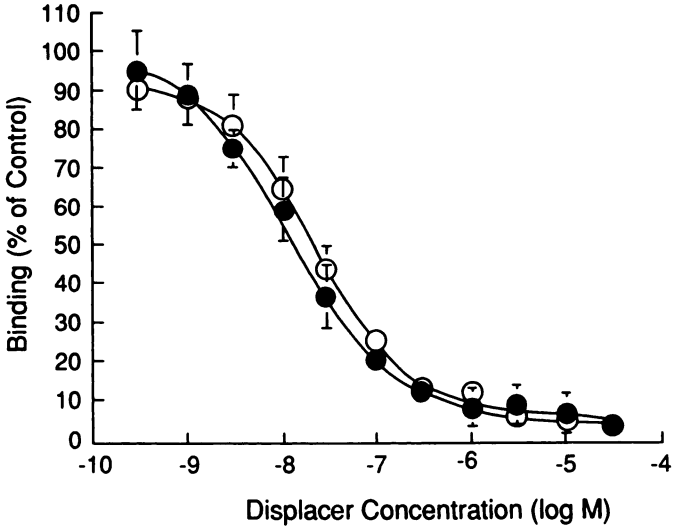

Fig. 3. Displacement of specific ^{125}I-angiotensin II binding by Dup-89 (○) in the presence of WL-19 (0.3 μM) and WL-19 (●) in the presence of Dup-89 (1 μM).

site having a high affinity for Dup-89 as site 1 and those binding sites having a high affinity for WL-19 as site 2.

In the absence of the antagonists, Scatchard analysis of ^{125}I-angiotensin II binding in the rat adrenal gave a K_d of 0.89 ± 0.11 nM and a maximal number of binding sites of 31 ± 5.8 pmol/g. In the presence of WL-19 or Dup-89 to occupy site 2 and site 1, Scatchard analysis of ^{125}I-angiotensin II binding indicated K_d values of 0.88 ± 0.12 and 1.1 ± 0.03 nM for site 1 and site 2, respectively, and a maximal number of binding sites of 18 ± 0.7 and 17 ± 1.0 pmol/g, respectively (data not shown).

Regional distribution of ^{125}I-angiotensin II binding sites 1 and 2 in the rat adrenal. Rat adrenals were dissected into capsular (mainly cortical glomerulosa cells) and decapsulated (remaining cortical cells and medulla) portions. In rat adrenal capsules, Dup-89 inhibited ^{125}I-angiotensin II binding with its high affinity site (site 1) accounting for approximately 60% of the total specific binding, whereas WL-19 inhibited approximately 40% of the total specific binding with high affinity (site 2) (Fig. 4A). In the presence of Dup-89 (1 μM) or WL-19 (0.3 μM) to occupy either site 1 or site 2, WL-19 and Dup-89 inhibited specific binding monophasically, with IC_{50} values of 23 ± 4 and 15 ± 1 nM, respectively, (Fig. 4A). In contrast, in the decapsulated adrenal, the relative amount of the total specific ^{125}I-angiotensin II binding that had high affinity for WL-19 was appreciably greater (~80%) than that for Dup-89 (~20%) (Fig. 4B). In the presence of 1 μM Dup-89 to occupy site 1, WL-19 displaced specific ^{125}I-angiotensin II binding monophasically, with an IC_{50} value of 44 ± 18 nM.

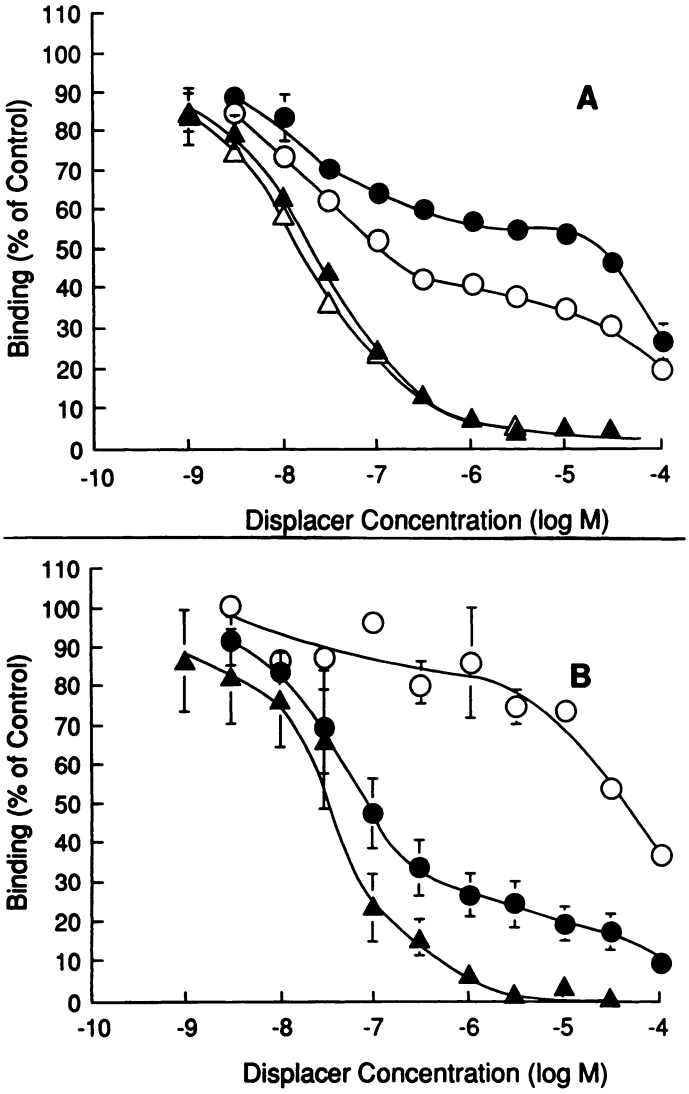

Fig. 4. Inhibition of specific ^{125}I-angiotensin II binding in rat adrenal capsules (A) and decapsulated tissues (B) by various concentrations of Dup-89 in the absence (○) or presence (△) of WL-19 (0.3 μM) or by WL-19 in the absence (●) or presence (▲) of Dup-89 (1 μM).

Effect of DTT on the inhibition of specific ^{125}I-angiotensin II binding in rat adrenal by Dup-89 and WL-19. Total specific ^{125}I-angiotensin II binding in rat whole adrenal membranes was reduced by 44 ± 2% when 5 mM DTT was present in the binding assay buffer. The displacement of ^{125}I-angiotensin binding by WL-19 in the presence of 5 mM DTT was monophasic with an IC_{50} value of 24 ± 8 nM, corresponding to its affinity for site 2 (Fig. 5). On the other hand, in the presence of DTT, Dup-89 also inhibited specific ^{125}I-angiotensin II binding, but with an IC_{50} value (110 ± 45 μM) corresponding to its low affinity for site 2 (Fig. 5).

Differential affinities of angiotensin III and Ile^7-angiotensin III but not angiotensin II and Sar^1,Ile^8-angiotensin II on ^{125}I-angiotensin II binding sites 1 and 2. To determine the affinities of these peptides for ^{125}I-angiotensin II binding sites 1 and 2 in rat whole adrenal, displacement studies with the peptides were performed in the presence of concentrations of Dup-89 or WL-19 to occupy site 1 and site 2, respectively. The results in Table 1 show that angiotensin II and Sar^1,Ile^8-angiotensin II have equal affinities for site 1 and site

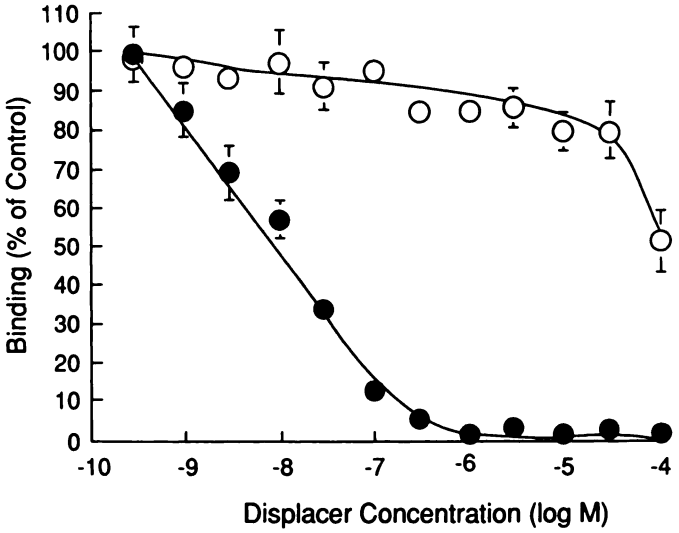

Fig. 5. Effect of DTT on the inhibition of specific ^{125}I-angiotensin II binding in rat whole adrenal by Dup-89 (○) and WL-19 (●).

TABLE 1

IC_{50} values for angiotensin II, angiotensin III, and peptide antagonists in displacing ^{125}I-angiotensin II binding in rat whole adrenal

	Site 1 + site 2[a]	Site 1[b]	Site 2[b]
Angiotensin II	0.73 ± 0.11	0.89 ± 0.033	0.73 ± 0.11
Antiotensin III	0.28 ± 0.10	1.3 ± 0.41	0.11 ± 0.032[c]
Sar^1,Ile^8-Angiotensin II	0.17 ± 0.022	0.18 ± 0.044	0.18 ± 0.015
Ile^7-Angiotensin III	0.28 ± 0.050	0.71± 0.075	0.12 ± 0.013[c]

[a] Neither Dup-89 or WL-19 were present int he incubation buffer.
[b] Determined in the presence of a sufficient concentration of WL-19 or Dup-89 to prevent binding to site 2 and site 1, respectively.
[c] $p < 0.05$, site 1 versus site 2.

2, whereas angiotensin III and Ile^7-angiotensin III had 12 and 6 times higher affinities, respectively, for site 2 compared with site 1.

Effect of Dup-89 and WL-19 on angiotensin II-stimulated aldosterone release in dispersed capsular cells. Dup-89 (300 nM) caused a parallel shift to the right of angiotensin II concentration-response curves, without significant reduction in the maximal response (Fig. 6). The inhibition of this angiotensin II response by Dup-89 was specific, in that at the same concentration Dup-89 has no effect on adrenocorticotropic hormone-stimulated aldosterone release in this preparation (data not shown). In contrast, WL-19 (1 μM) had no effect on angiotensin II-stimulated aldosterone release.

Discussion

The present studies indicate that specific ^{125}I-angiotensin II binding in the rat whole adrenal represents binding to two distinct sites. This view is supported by the finding that the nonpeptide angiotensin II receptor ligands Dup-89 and WL-19 displaced specific ^{125}I-angiotensin II binding in a biphasic manner. Moreover, in the presence of Dup-89 or WL-19 to prevent binding to their high affinity sites, the remainder of the ^{125}I-angiotensin II specifically bound was displaced by WL-19 or Dup-89, respectively, in a monophasic manner and with high affinity. The results indicate that the high affinity binding sites for Dup-89 and WL-19 are distinct sites and correspond to the low affinity site of the other. We have designated the sites having high affinity for Dup-89 as site 1 and those having a high affinity for WL-19 as site 2. The view that site 1 and site 2 represent two separate receptor sites without cooperative interaction was further indicated by kinetic studies showing that neither Dup-89 nor WL-19 affect the dissociation rate of angiotensin II from its receptors (data not shown). The present data provide strong support for several previous proposals for the existence of angiotensin receptor subtypes (1–5).

^{125}I-Angiotensin II binding sites 1 and 2 could also be differentiated using the disulfide-reducing agent DTT. The presence of DTT in the incubation medium prevented the binding of ^{125}I-angiotensin II to site 1, as indicated by the low affinity of Dup-89 and the high affinity of WL-19 in displacing the bound ligand under these conditions. The data indicate that intact disulfide bonds are essential for the binding of ^{125}I-angiotensin II to site 1 in the rat adrenal. DTT has also previously been reported to reduce specific ^{125}I-angiotensin II binding in bovine adrenal cortex (14).

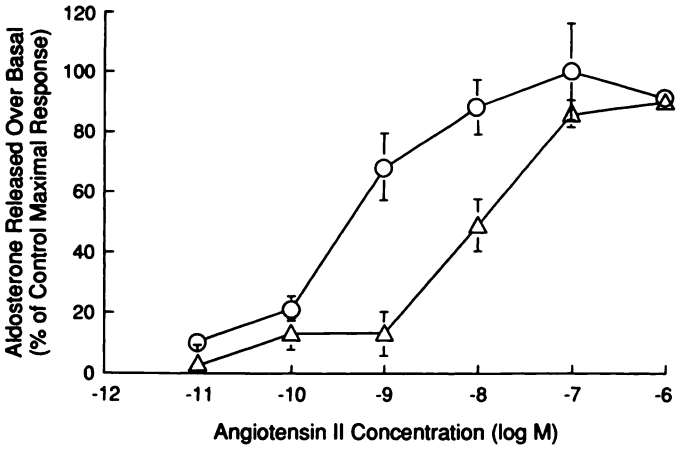

Fig. 6. Inhibition of angiotensin II-induced aldosterone release by Dup-89 in rat adrenal capsular cells. ○, Control; △, treated with Dup-89 (300 nM).

The correlation of the presently described ^{125}I-angiotensin II binding sites 1 and 2 with the previously described distinct receptors for angiotensin II and angiotensin III in adrenal tissue (see Introduction) is not clear. However, it is interesting to note that, whereas angiotensin II and Sar^1,Ile^8-angiotensin II have the same affinity for site 1 and site 2, angiotensin III and Ile^7-angiotensin III have 6–12 times higher affinity for site 2 than site 1. Moreover, angiotensin III has 6 times higher affinity for site 2 than angiotensin II. The affinity of angiotensin III for site 2 (0.11 nM) was remarkably similar to the high affinity angiotensin III binding site reported by others in the rat adrenal (11), using [^{3}H]angiotensin III as the ligand. Thus, it is tempting to relate site 2 to the previously described specific angiotensin III receptor in rat adrenal reported by some investigators. The differential affinities of the angiotensin II analogs for site 1 and site 2 were also observed in rat brain and monkey kidney.[1]

In functional studies using adrenal capsular cells, angiotensin II-stimulated aldosterone release was effectively antagonized by Dup-89 in a manner consistent with competitive inhibition, whereas WL-19 was inactive. Based upon the shift in the angiotensin II concentration-response curve in the absence and presence of Dup-89, the estimated pA_2 value for Dup-89 was 7.9 (K_B = 13 nM). This value is in good agreement with the affinity of Dup-89 for site 1 (15 nM) determined in binding studies using adrenal cortical tissue. Thus, the ^{125}I-angiotensin II binding site 1, but not site 2, appears to be involved in mediation of angiotensin II-stimulated aldosterone release.

The function of the high affinity ^{125}I-angiotensin II binding sites for WL-19 (site 2) remains to be determined. Angiotensin II-mediated contractions of the rat pulmonary artery were previously reported to be antagonized by Dup-89, but not WL-19, suggesting that the vascular contractions produced by angiotensin II are also mediated by an action upon ^{125}I-angiotensin II binding site 1 (2). This view is supported by the finding that DTT, which prevents binding of ^{125}I-angiotensin II to site 1 (see above), has been reported to specifically inhibit contractile responses of the isolated rabbit aorta produced by angiotensin II (16).

The majority of ^{125}I-angiotensin II binding sites in the decapsulated rat adrenal have high affinity for WL-19. Because autoradiographic studies using ^{125}I-labeled angiotensin II or III show that much higher concentrations of angiotensin receptors occur in the zona glomerulosa and medulla than in intermediate cortical layers (17, 18), the majority of angiotensin II binding sites in the decapsulated rat adrenal most likely represent binding to medullary tissue. It is possible that site 2 may play a role in mediating angiotensin II-induced release of catecholamines from the adrenal medulla (19). In this regard, ^{125}I-angiotensin II binding site 2 is also abundant in rat brain, another neuronal tissue (2).

Recent studies in our laboratory have shown that the presence and proportion of ^{125}I-angiotensin II binding sites 1 and 2 vary greatly within different tissues of the same species and among the same tissues of different species and this will be the subject of a subsequent report.

Since this manuscript was submitted and reviewed, White-

[1] Unpublished observations.

bread *et al.* (20) have similarly presented evidence for the preferential affinity of the nonpeptide DuPont antagonist and a peptide antagonist for distinct angiotensin receptor subtypes.

Acknowledgments

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