0026-895X/91/030401-06$03.00/0

MOLECULAR PHARMACOLOGY, 40:401-406

Angiotensin II Receptor Subtypes and Biological Responses in the Adrenal Cortex and Medulla

TAMAS BALLA, ALBERT J. BAUKAL, SAMUEL ENG, and KEVIN J. CATT

Endocrinology and Reproduction Research Branch, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892.

Received January 2, 1991; Accepted June 10, 1991

SUMMARY

Angiotensin II (AII) receptor subtypes and their potential coupling mechanisms were studied using recently developed peptide and nonpeptide antagonists in rat and bovine adrenal zona glomerulosa cells, as well as in membranes prepared from rat and bovine adrenal cortex and medulla. Comparison of the potencies of these novel antagonists to displace ^{125}I-[Sar^1,Ile^8]AII from its binding sites revealed two distinct AII binding sites in membranes prepared from rat adrenal capsules (zona glomerulosa) and from rat adrenal inner zones containing the medulla. About 85% of the binding sites of the glomerulosa zone and 30% of those of the inner zones were of the AT_1 subtype, with relative affinities for the nonpeptide antagonists Dup 753 and PD 123177 and the peptide antagonist CGP 42112A in the order of Dup 753 ≫ CGP 42112A > PD 123177. In contrast, the relative binding potencies for the other (AT_2) population of binding sites were CGP 42112A > PD 123177 ≫ Dup 753. Neither AII nor its peptide antagonist [Sar^1,Ile^8]AII could distinguish between the two sets of binding sites. The effects of the new antagonists on functional responses of rat adrenal glomerulosa cells demonstrated that both AII-stimulated aldosterone production and the AII-induced inhibition of adrenocorticotropic hormone-stimulated cAMP formation were mediated by the AT_1 receptor subtype. In bovine adrenals, only AT_1 receptors were detected in membranes prepared from the cortex and the medulla, as well as in cultured glomerulosa cells. The relative inhibitory potency of Dup 753 was lower by an order of magnitude at bovine than at rat AT_1 receptors. The inhibition of AII-induced aldosterone production by the various antagonists was closely correlated with their inhibitory potencies on ^{125}I-[Sar^1,Ile^8]AII binding to bovine glomerulosa cells. These data suggest that the known effects of AII in adrenal glomerulosa cells are mediated through the AT_1 receptor subtype and that the distribution and/or specificity of the AT_2 receptors shows marked species variations.

AII, a potent vasoconstrictor peptide and a major regulator of mineralocorticoid secretion from the adrenal gland, exerts its biological effects through binding to specific receptors in the plasma membrane of its numerous target cells. Several studies on adrenal, liver, and smooth muscle have suggested that, unlike many other cell surface receptors, there is only a single population of AII receptors (1–5); however, others have indicated the possibility of AII receptor subtypes, based on functional responses (6–8). It is well documented that AII receptors belong to the class of Ca^{2+}-mobilizing receptors that activate a phosphoinositide-specific phospholipase C, acting through a pertussis toxin-insensitive putative G protein (see Ref. 9 for review). In many target tissues, AII also inhibits adenylate cyclase through another G protein, which is sensitive to pertussis toxin and is apparently a member of the G_i family (8, 10, 11). Other effects of AII on second messenger formation, such as activation of calcium channels and stimulation of Ca^{2+} influx, have also been reported to be sensitive to pertussis toxin (12, 13). These data suggest that AII receptors are coupled to several different transmembrane signaling pathways and raise the possibility that subpopulations of AII receptors exist, as described in the case of many other cell surface receptors.

The recent development of novel peptide (14) and nonpeptide (15, 16) AII antagonists has unequivocally demonstrated the existence of at least two distinct subpopulations of AII receptors, based on ligand binding studies (14, 15). Unlike the natural ligand AII and most of its numerous peptide agonist and antagonist derivatives, these new antagonists exhibit selectivity for the two major receptor subtypes. The development of such compounds has made it possible to determine whether different receptor subtypes are related to the various coupling mechanisms described above.

In the present study, we compared the binding-inhibition potencies of these new antagonists in rat and bovine adrenal membrane preparations and measured the distribution of the receptor subpopulations between the cortex and medulla. We also defined the pharmacology of specific AII-stimulated responses in rat and bovine adrenal glomerulosa cell preparations. These studies have shown that all AII-evoked responses tested in the adrenal gland are mediated solely by the AT_1 receptor

ABBREVIATIONS: AII, angiotensin II; G protein, guanine nucleotide-binding protein; M-199, medium 199; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ACTH, adrenocorticotropic hormone.

population and that the function of the AT_2 receptor subtype has yet to be determined.

Materials and Methods

Preparation of adrenal membranes. Particulate adrenal fractions were prepared from tissues as previously described (4). Briefly, fresh bovine adrenals were trimmed of fat and, after bisection, the medulla was separated from the cortex by scraping. The respective tissues were minced and homogenized in 20 mM $NaHCO_3$, using a Dounce tissue homogenizer, and filtered through nylon mesh. The homogenate was centrifuged at 1,400 × *g* and the supernatant was recentrifuged at 20,000 × *g*. The resulting particulate fraction was washed with binding buffer (20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.2% bovine serum albumin, 0.002% NaN_3) and finally resuspended in the same buffer at a concentration of 1–2 mg of protein/ml. When adrenals from male Sprague-Dawley rats were used, the animals were decapitated and the adrenals were removed and freed of fat. The adrenal capsules (zona glomerulosa) were separated from the decapsulated tissue (inner zones plus medulla) by the method of Giroud *et al.* (17). Membranes were then prepared according to the protocol described above.

Preparation of adrenal glomerulosa cells. Rat adrenal glomerulosa cells were prepared from the capsular tissue (zona glomerulosa) of the adrenals of male Sprague Dawley rats (220–280 g), as previously described (8). Cells were kept at room temperature in modified M-199, Hanks' buffered salt solution (3.6 mM K^+, 1.2 mM Ca^{2+}, 25 mM HCO_3^-, 5 mM HEPES, pH 7.4), containing 0.2% bovine serum albumin (incubation medium), for 2–3 hr before final incubations.

Bovine adrenal glomerulosa cells were prepared and cultured as described previously (18), except that metopyrone (5 μM) was present during the culture period. Cells were kept without serum for 1 day before use in binding studies or aldosterone production experiments, which were performed on the third or fourth day of culture.

Binding studies on adrenal membranes and cells. Adrenal membranes (cortex or medulla; about 50–100 μg of protein) were incubated in 200 μl final volume of binding buffer (see above) containing ^{125}I-[Sar^1,Ile^8]AII (0.03 μCi/tube) and the indicated concentrations of one of the unlabeled agonist or antagonist ligands. Incubations were performed for 30 min at room temperature, and the membrane-bound radioactivity was separated from the unbound ligand on Whatman GF/B filters with a Brandel vacuum filtration apparatus.

When binding to cultured bovine adrenal cells was studied, cells grown in 24-well plates were incubated in 0.5 ml of the modified M-199 (see above, except that 20 mM HEPES, pH 7.4, was used for buffering without $NaHCO_3$) in the presence of ^{125}I-[Sar^1,Ile^8]AII (0.03 μCi/well) and the desired final concentration of the respective antagonist. Incubations were performed at room temperature for 60 min. Cells were then washed on ice with 2 × 0.5 ml of the same medium (without tracer and antagonists) and dissolved in 0.01% sodium dodecyl sulfate, 0.5 M NaOH (2 × 200 μl).

Incubations for aldosterone production. Freshly prepared rat adrenal glomerulosa cells were kept at room temperature for 3 hr before use, centrifuged, and resuspended in bicarbonate-free, HEPES-buffered modified M-199 (see above). About 10^5 cells were incubated in 1-ml volume in the presence of 10^{-8} M AII, with or without selected concentrations of the antagonists, for 60 min at 37° under a CO_2/O_2 (95:5) atmosphere. Cells were then placed on ice and subsequently centrifuged; the supernatant was analyzed for aldosterone content, using an ^{125}I-aldosterone radioimmunoassay kit (Coat-A-Count; Diagnostics Products Corp., Los Angeles, CA).

Before aldosterone experiments, cultured bovine adrenal glomerulosa cells were washed and incubated for 1 hr in bicarbonate-buffered modified M-199 at 37°, to remove metopyrone. The medium was then replaced and the cells (10^6/well) were stimulated with AII (3×10^{-9} M), with or without the antagonists, for 2 hr in an air/CO_2 incubator. Cells were then placed on ice, and the medium was removed for aldosterone analysis.

Measurement of cAMP in rat adrenal glomerulosa cells. For cAMP measurements, rat adrenal cells (2.5×10^5 cell/tube) were incubated in 0.5 ml of bicarbonate-free, HEPES-buffered modified M-199, in the presence of 1 mM isomethylbutylxanthine, at 37°. Cells were preincubated for 15 min with or without AII (3×10^{-8} M), in the presence or absence of one of the AII antagonists, and then ACTH (10^{-8} or 10^{-10} M) was added for 10 min. Incubations were terminated by placing the tubes in boiling water for 10 min, followed by sonication. After centrifugation, the cAMP content of the supernatant was determined by radioimmunoassay after an acetylation step (19).

Data analysis. The IC_{50} values in the binding-inhibition studies were determined by analyzing the family of dose-displacement curves from each individual experiment with the four-parameter logistic model, using the FLEXIFIT program (20). In composite curves (rat adrenal cortex and medulla), the individual components were analyzed after graphical determination of the inflection point. The dissociation constant (K_d) and the density of the binding sites (B_{max}) were determined by Scatchard analysis of the displacement curves using [Sar^1,Ile^8]AII as the ligand. Statistical comparisons were made using Student's *t* test.

Materials. ^{125}I-[Sar^1,Ile^8]AII and ^{125}I-AII specific activity, (1800 Ci/mmol) were obtained from Hazelton Laboratories (Vienna, VA). [Sar^1,Ile^8]AII was purchased from Peninsula Laboratories (Belmont, CA). The peptide antagonist nicotinic acid-Tyr-(N^α-benzyloxycarbonyl-Arg)Lys-His-Pro-Ile-OH (CGP 42112A) was provided by Dr. M. de Gasparo (CIBA-GEIGY, Basel, Switzerland). The nonpeptide antagonists Dup 753 and PD 123177 (previously called EXP 655) were provided by Dr. P. C. Wong (DuPont, Wilmington, DE). Materials used for cell isolation and incubations have been described previously (8, 18).

Results

Binding studies on adrenal membranes. The relative potencies for inhibition of binding of ^{125}I-[Sar^1,Ile^8]AII by the novel peptide antagonist CGP-42112A and two nonpeptide antagonists (Dup 753 and PD 123177), as well as [Sar^1,Ile^8]AII and AII itself, were analyzed in membranes prepared from both rat and bovine adrenal cortex and medulla. In membranes prepared from rat adrenal capsules (zona glomerulosa), [Sar^1,Ile^8]AII showed the highest potency in inhibiting specific binding of the homologous radioligand, followed closely by the natural agonist AII, with <0.5 log unit difference between the IC_{50} values (Fig. 1, *upper*; Table 1). The nonpeptide antagonist Dup 753 was almost as potent as AII in displacing about 80–85% of the radioligand. However, the rest of the binding was resistant to the antagonist and required concentrations higher than 10^{-4} M for complete displacement. In contrast, the other nonpeptide antagonist, PD 123177, displaced about 15% of the specific binding at low concentrations (IC_{50} about 5×10^{-8} M) and inhibited the remainder of the binding only at very high concentrations (above 3×10^{-5} M). The novel peptide antagonist CGP 42112A was as potent as [Sar^1,Ile^8]AII in displacing the radioligand from the minor subpopulation (AT_2) and was less potent than the Dup 753 compound in displacing the majority of the binding (AT_1) (Fig. 1, *upper*; Table 1).

When the binding to decapsulated rat adrenal tissue (inner cortical zones plus medulla) was studied, the findings differed, in that the major portion of the binding (about 70%) was displaced by low concentrations of the PD 123177 and CGP 42112A compounds and the Dup 753-sensitive component represented only about 30% of the binding sites (Fig. 1, *lower*). These data are consistent with previous reports (15) of two different binding sites (AT_1 and AT_2) in the rat adrenal gland and their relative distributions between the zona glomerulosa and the adrenal medulla.

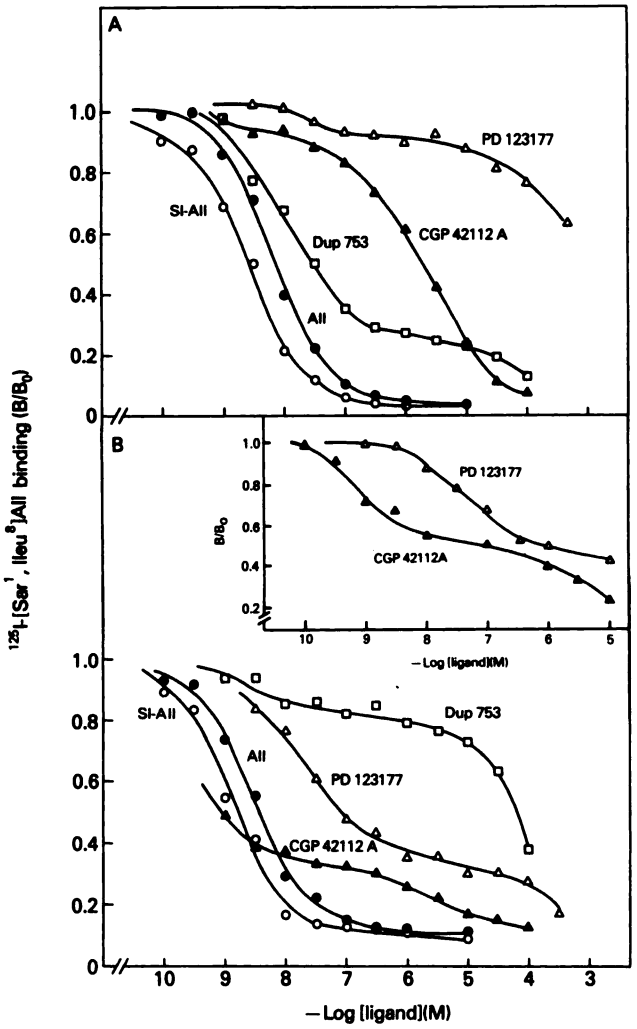

Fig. 1. Inhibition by AII (●), [Sar^1,Ile^8]AII (*SI-AII*) (○), Dup 753 (□), PD 123177 (△), and CGP 42112A (▲) of ^{125}I-[Sar^1,Ile^8]AII binding in membranes prepared from rat adrenal glomerulosa tissue (A) and from rat adrenal inner zones (B). Each *point* represents the mean of duplicate determinations. Similar results were obtained in two other independent experiments using different membrane preparations. *Inset* in B, full dose-displacement curves for CGP 42112A (▲) and PD 123177 (△) in another rat adrenal medullary membrane preparation.

In contrast, adrenocortical membranes prepared from bovine adrenals did not show the presence of the two distinct AII binding sites observed in the rat. As shown in Fig. 2 (*upper*), ^{125}I-[Sar^1,Ile^8]AII was displaced by each of the ligands tested in a manner characteristic of the AT_1 receptor sites. The relative potencies of the different antagonists were in the same order but showed significant differences from those observed in the rat adrenal glomerulosa for the major subpopulation of the binding sites. The potencies of [Sar^1,Ile^8]AII and Dup 753 to inhibit ^{125}I-[Sar^1,Ile^8]AII binding to bovine adrenals differed by 2 log units, in contrast to the <1 log unit difference found in the rat adrenal (see Table 1).

The binding properties of membranes prepared from bovine adrenal medulla were similar to those observed in the adrenal cortex. There was no evidence for the presence of binding sites insensitive to Dup 753, and the relative displacement potencies of the different ligands were the same as in bovine adrenocortical membranes (Fig. 2, *lower*). The displacement curves were similar when ^{125}I-AII was used as the tracer in the binding experiments (data not shown). When dithiothreitol (5 mM) was included in the buffer of binding assays performed on bovine adrenal cortex or medulla, the total amount of binding was substantially decreased (by 43% and 59%, respectively) but no change was observed in the displacement curves for the AII antagonists (data not shown).

Aldosterone production by zona glomerulosa cells. The inhibitory effects of the several AII antagonists on AII-stimulated aldosterone production were studied in freshly prepared rat adrenal glomerulosa cells and in cultured bovine adrenal glomerulosa cells. As shown in Fig. 4 (*upper*), [Sar^1,Ile^8]AII and the Dup 753 and CGP 42112A antagonists completely inhibited the stimulatory effect of AII (10^{-8} M) on aldosterone production, with relative potencies that matched their abilities to displace the radioligand from the major population of binding sites described in rat adrenal glomerulosa membranes (see above). On the other hand, the PD 123177 antagonist had no effect on AII-stimulated aldosterone production, up to a concentration of 10^{-5} M.

In cultured bovine adrenal glomerulosa cells, AII-stimulated aldosterone production was inhibited by the antagonists with potencies similar to those observed in the radioligand binding experiments (compare Figs. 2 and 3).

Effects of antagonists on AII-induced inhibition of cAMP production in rat adrenal glomerulosa cells. The effects of the different antagonists on the inhibition by AII of ACTH-stimulated cAMP production were analyzed in rat adrenal glomerulosa cells. Both [Sar^1,Ile^8]AII and Dup 753 reversed the AII-induced inhibition of cAMP production at concentrations sufficient to inhibit radioligand binding to the major population of AII binding sites (AT_1). In contrast, neither PD 123177 nor the CGP 42112A antagonist restored the cAMP response at concentrations that displaced radioligand binding from the minor (AT_2) population of receptor sites but did not affect binding to the major (AT_1) proportion. In three experiments, the cAMP responses to 10^{-8} M ACTH were significantly decreased by 3×10^{-8} M AII ($p < 0.05$) or by the combinations of AII and 10^{-5} M PD 123177 ($p < 0.005$) or AII plus 10^{-7} M CGP 42112A ($p < 0.025$) but were not significantly affected by the combinations of AII and 10^{-6} M [Sar^1,Ile^8]AII or AII and 10^{-5} M Dup 753 (Fig. 4, *lower*).

Discussion

This study was undertaken to explore the functional relevance of the two subpopulations of AII receptors recently identified by the use of novel selective peptide and nonpeptide antagonists (14–16). We intended to employ cultured bovine adrenal glomerulosa cells for this purpose, because we have characterized many AII-evoked responses in this preparation (21, 22). However, binding-inhibition studies with several antagonists revealed the presence of only one type of AII binding sites in these cells, corresponding to the AT_1 receptor. A comparison of the potencies of the antagonists to displace radioligand from the binding sites and to inhibit AII-stimulated aldosterone production indicated that the single AT_1 binding site population is responsible for mediating the stimulation of aldosterone secretion.

To exclude the possibility that the AT_2 binding sites could be present but lost during cell isolation and culture, we evaluated the binding properties of freshly prepared bovine adrenocortical membranes as well as membranes prepared from the adrenal medulla. These studies showed only the same population of AII binding sites (AT_1) that was observed in the cultured

TABLE 1

Inhibitory constants (IC_{50}) of individual ligands for displacement of ^{125}I-[Sar^1,Ile^8]AII binding in rat and bovine adrenal membranes

Values are mean ± standard error from three determinations

	Cortex	Medulla
Rat adrenal		
IC_{50} values (M)		
[Sar^1,Ile^8]AII	$2.8 \pm 0.43 \times 10^{-9}$	$2.6 \pm 1.15 \times 10^{-9}$
AII	$6.6 \pm 0.20 \times 10^{-9}$	$4.9 \pm 1.86 \times 10^{-9}$
Dup 753 (AT_1)	$1.3 \pm 0.19 \times 10^{-8}$	$1.6 \pm 0.51 \times 10^{-8}$
Dup 753 (AT_2)	$7.4 \pm 0.09 \times 10^{-5}$	$8.2 \pm 0.94 \times 10^{-5}$
CGP 42112A (AT_2)	$2.1 \pm 0.55 \times 10^{-9}$	$6.1 \pm 3.21 \times 10^{-10}$
CGP 42112A (AT_1)	$2.5 \pm 0.55 \times 10^{-6}$	$8.4 \pm 3.44 \times 10^{-6}$
PD 123177 (AT_2)	$4.9 \pm 0.90 \times 10^{-8}$	$1.0 \pm 0.40 \times 10^{-7}$
PD 123177 (AT_1)	$4.5 \pm 1.96 \times 10^{-4}$	$2.1 \pm 0.72 \times 10^{-4}$
[Sar^1,Ile^8]AII binding parameters		
K_{d_1} (M)	$1.4 \pm 0.21 \times 10^{-9}$	$1.2 \pm 0.07 \times 10^{-9}$
K_{d_2} (M)	$6.6 \pm 0.67 \times 10^{-8}$	$5.5 \pm 1.40 \times 10^{-8}$
B_{max_1} (fmol/mg of protein)	764 ± 211	47 ± 10
B_{max_2} (fmol/mg of protein)	2820 ± 590	403 ± 52
Bovine adrenals		
IC_{50} values (M)		
[Sar^1,Ile^8]AII	$6.2 \pm 1.31 \times 10^{-9}$	$2.0 \pm 0.32 \times 10^{-9}$
AII	$4.9 \pm 0.85 \times 10^{-8}$	$2.0 \pm 0.15 \times 10^{-8}$
Dup 753	$7.3 \pm 1.38 \times 10^{-7}$	$2.5 \pm 0.42 \times 10^{-7}$
CGP 42112A	$8.0 \pm 3.06 \times 10^{-6}$	$3.6 \pm 0.15 \times 10^{-6}$
PD 123177	$1.1 \pm 0.01 \times 10^{-3}$	$7.0 \pm 0.85 \times 10^{-4}$
[Sar^1,Ile^8]AII binding parameters		
K_{d_1} (M)	$4.3 \pm 1.50 \times 10^{-10}$	$3.8 \pm 1.42 \times 10^{-10}$
K_{d_2} (M)	$1.5 \pm 1.21 \times 10^{-8}$	$3.0 \pm 0.94 \times 10^{-8}$
B_{max_1} (fmol/mg of protein)	537 ± 144	172 ± 20
B_{max_2} (fmol/mg of protein)	1431 ± 122	826 ± 290

glomerulosa cells. It has been shown that dithiothreitol inhibits AII binding to the AT_1 receptors in the rat adrenal cortex and, conversely, enhances AII binding to the minor population of AT_2 sites (14, 23). In bovine adrenal membranes prepared from either the cortex or the medulla, dithiothreitol inhibited the binding of ^{125}I-[Sar^1,Ile^8]AII, and there was no evidence for the presence of AT_2 receptors under these conditions. However, it should be noted that Scatchard analysis of the radioligand binding-inhibition data for AII and [Sar^1,Ile^8]AII indicates the presence of two subpopulations of AT_1 sites, with affinities differing by 1.5–2 log units, in both rat and bovine adrenal membranes.

In further experiments, rat adrenals were analyzed for the presence of the AT_1 and AT_2 receptor subpopulations. In accordance with previous findings (15), we observed that most of the AII binding (about 85%) in the rat zona glomerulosa was to the AT_1 sites but about 15% of the binding sites showed a pharmacology characteristic of the AT_2 sites. Conversely, in the rat adrenal medulla, the major proportion of the AII binding sites was found to be the AT_2 type and the smaller proportion to be the AT_1 type. [Because the zona fasciculata-reticularis contains negligible AII binding sites in the rat (15, 24), AII binding to the decapsulated adrenals is due to receptors in the medullary tissue.] Recently, it has been reported that AII-induced epinephrine secretion from perfused rat adrenals was mediated via the small population of AT_1 receptors and not through the abundant AT_2 subtype (25). The pharmacology of the AII-stimulated aldosterone response of rat adrenal glomerulosa cells also showed that, even if present, the AT_2 sites are not linked to any mechanism that would stimulate aldosterone secretion. Thus, selective blockade of the AT_1 but not the AT_2 sites completely abolished the stimulatory effect of AII on aldosterone secretion.

AII receptors in the rat adrenal zona glomerulosa have been shown to be coupled to both the G_i and putative G_p (or G_q) proteins to inhibit adenylate cyclase and stimulate phosphoinositide hydrolysis, respectively (8, 11, 26, 27). Therefore, a possible function of the AT_2 sites in the rat zona glomerulosa could be to inhibit adenylate cyclase, analogous to the function of the α_2-adrenergic receptors. We found, however, that the inhibitory effect of AII on ACTH-stimulated cAMP formation was not blocked by the PD 123177 and CGP 42112A compounds at concentrations that are selective for the AT_2 sites but was completely inhibited by blockade of the AT_1 receptors. Thus, the inhibition of adenylate cyclase enzyme by AII is not mediated by the AT_2 receptors present in the rat adrenal glomerulosa cells. This finding raises the possibility that AT_1 receptors, like tha α-adrenergic receptors, have additional subtypes that could account for the better fit by a two-site model in the Scatchard analysis of the binding-inhibition data.

Comparison of the binding data obtained in the bovine and rat adrenal cortex and medulla reveals several notable species differences. The most remarkable of these is the lack of AT_2 sites in the bovine adrenal cortex and, even more so, in the medulla. This is not due to the lack of this receptor type in the cow, because AT_2 binding sites have been shown to be present in other bovine tissues such as the bovine cerebellum.[1] It is also worth noting that the bovine AT_1 receptors distinguish more clearly between [Sar^1,Ile^8]AII, AII, and Dup 753, showing a difference of about 1 order of magnitude between their IC_{50} values. In contrast, the affinities of these ligands for the rat AT_1 receptor are very close, with <1 log unit difference between the IC_{50} values for [Sar^1,Ile^8]AII and Dup 753. A similar difference between the bovine and rat AT_1 receptors in their ability to discriminate between [Sar^1,Ile^8]AII and Dup 753 is apparent from the comparison of the relative potencies of the two antag-

[1] R. Speth, personal communication.

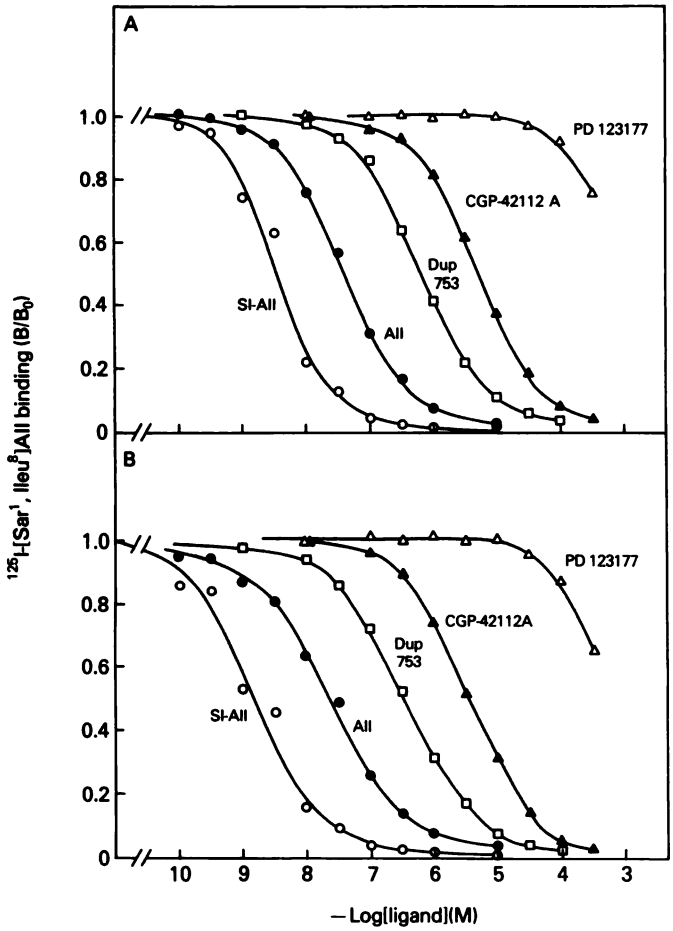

Fig. 2. Inhibition by AII, [Sar^1,Ile^8]AII (*SI-AII*), Dup 753, PD 123177, and CGP 42112A of ^{125}I-[Sar^1,Ile^8]AII binding in membranes prepared from bovine adrenal glomerulosa tissue (A) and from bovine adrenal medulla (B). Each *point* represents the mean of duplicate determinations. Similar results were obtained in three experiments using different membrane preparations.

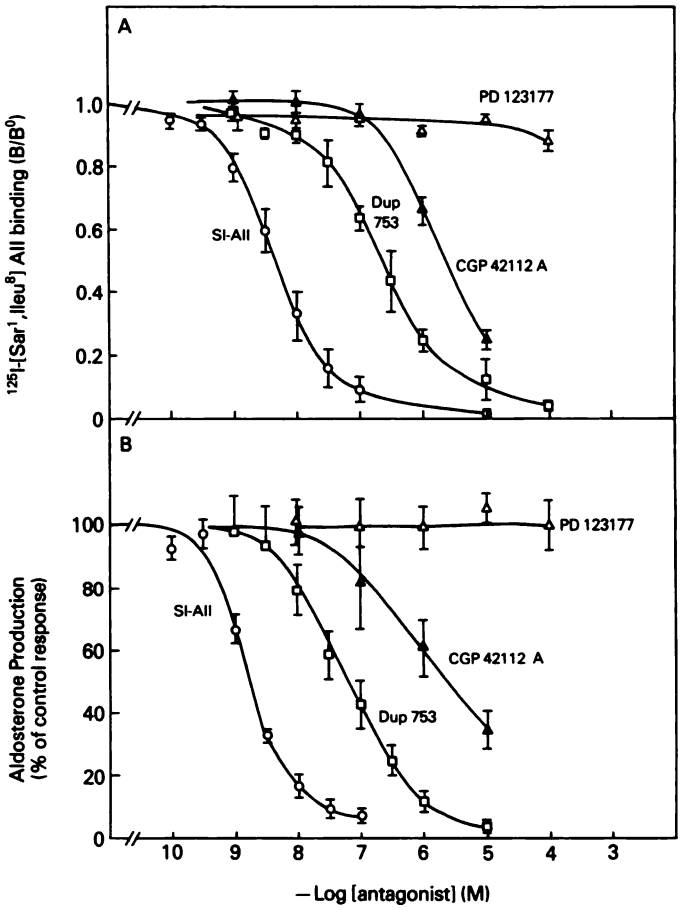

Fig. 3. Inhibition by [Sar^1,Ile^8]AII (*SI-AII*), Dup 753, PD 123177, and CGP 42112A of ^{125}I-[Sar^1,Ile^8]AII binding in cultured bovine adrenal glomerulosa cells (A), and inhibition of the aldosterone secretory response of the cells to AII (3×10^{-9} M) by the same compounds (B). The means ± standard errors of four to six points obtained in separate experiments are shown (except for the curve for PD 123177 in A, where only two or three determinations were made). The average basal aldosterone production was 35.6 ± 4.6 pg/10^6 cells/2 hr (mean ± standard error, 10 experiments, which was increased 54 ± 8-fold (mean ± standard error) in response to 3×10^{-9} M AII.

onists to inhibit aldosterone secretion. This species difference between the binding characteristics of the AT_1 receptors may be relevant to the size difference between the photoaffinity-labeled AII receptors of the rat and bovine adrenals (28). These differences in the binding characteristics of the rat and bovine AII receptors led us to test the sensitivity to the several AII antagonists of other AII-induced responses of bovine adrenal cells (including the ability to increase cytosolic Ca^{2+}, stimulate c-*fos* expression, and induce changes in the highly phosphorylated inositols, an effect that requires prolonged exposure to AII); all of these responses were inhibited by blockade of the AT_1 receptor sites.[2]

In this report, we have compared the relative affinities and potencies of novel AII antagonists that have not previously been compared in one study using the same membrane or cell preparations. Both the CGP 42112A and the PD 123177 compounds showed high selectivity for the AT_2 site. The CGP 42112A compound, which possesses very high affinity for the AT_2 site (IC_{50} of about 10^{-9} M) is also effective on the AT_1 site at relatively low concentrations, unlike the PD 123177 compound, which must be used at very high concentrations (over 10^{-4} M) to inhibit the AT_1 receptor. Consistent with earlier reports (15), the Dup 753 compound showed great selectivity for the AT_1 site and only at high concentrations (above 10^{-5} M) displaced binding from the AT_2 receptor site. In a recent study using the same antagonists, the pharmacology of the amphibian AII receptor was found to be strikingly different from that of the mammalian receptor (29). However, apart from the species differences noted above, we found the relative potencies of these compounds to be in the same order in rat and bovine adrenal glands.

In summary, two AII receptor subtypes were detected in the rat adrenal gland, but only one of these was present in the bovine adrenal cortex and medulla. The known effects of AII on adrenal function, such as stimulation of aldosterone production and inhibition of adenylate cyclase, were mediated by the AT_1 receptor site. No function has been found to be attributable to the AT_2 sites, which are more abundant in the rat adrenal medulla. These data are consistent with the recent findings that the contractile actions of AII, as well as its stimulatory effect on adrenal epinephrine secretion, are mediated by the small population of AT_1 sites present in rat uterus and adrenal medulla, respectively, where most of the receptors are the AT_2 subtype (25, 30). The nature and the function of the AT_2

[2] T. Balla, A. Clark, C. Ambroz, A. J. Baukal, and K. J. Catt, unpublished observations.

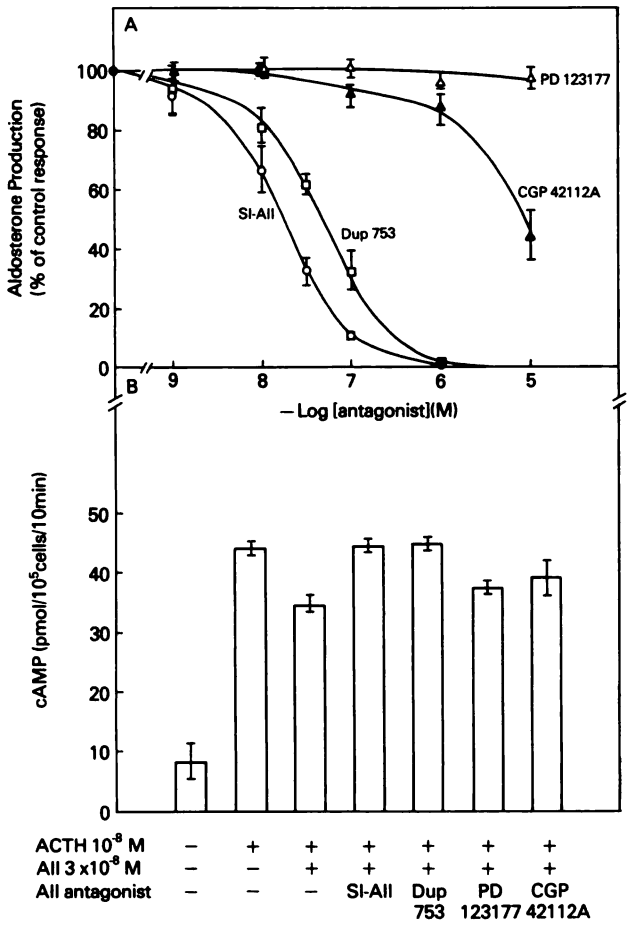

Fig. 4. A, Inhibition by AII antagonists of the aldosterone secretory response of rat adrenal glomerulosa cells to AII (10^{-8} M). Means ± standard errors of four experiments are shown. The average basal aldosterone production was 182.4 ± 30.9 pg/2.5 × 10^5 cells/60 min, with a 8 ± 1.7-fold increase in response to 10^{-8} M AII. B, Effects of the AII antagonists on the inhibitory action of AII on ACTH-stimulated cAMP production in rat adrenal glomerulosa cells. The concentrations of the antagonists were [Sar^1,Ile^8]AII (*SI-AII*), 10^{-6} M; Dup 753 and PD 123177, 10^{-5} M; and CGP 42112A, 10^{-7} M. Means ± standard errors of triplicate determinations are shown, from one of three experiments with identical results.

receptors, which do not appear to interact with G proteins (30, 31), remain to be clarified.

References

1. Glossmann, H., A. Baukal, and K. J. Catt. Angiotensin II receptors in bovine adrenal cortex: modification of angiotensin II binding by guanyl nucleotides. *J. Biol. Chem.* **249**:664–666 (1974).
2. Capponi, A. M., and K. J. Catt. Angiotensin II receptors in adrenal cortex and uterus: binding and activation properties of angiotensin analogues. *J. Biol. Chem.* **254**:5120–5127 (1979).
3. Guillemette, G., G. Guillon, J. Marie, M. N. Balestre, E. Escher, and S. Jard. High yield labeling of angiotensin II receptors. *Mol. Pharmacol.* **30**:544–551 (1986).
4. Glossmann, H., A. Baukal, G. Aguilera, and K. J. Catt. Radioligand assay for angiotensin II receptors. *Methods Enzymol.* **109**:110–126 (1985).
5. Bouscarel, B., P. F. Blackmore, and J. H. Exton. Characterization of the angiotensin II receptor in primary cultures of rat hepatocytes. *J. Biol. Chem.* **263**:14913–14919 (1988).
6. Wright, G. B., R. W. Alexander, L. S. Ekstein, and M. A. Gimbrone, Jr. Characterization of the rabbit ventricular myocardial receptor for angiotensin II: evidence for two sites of different affinities and specificities. *Mol. Pharmacol.* **24**:213–221 (1983).
7. Gunther, S. Characterization of angiotensin II receptor subtypes in rat liver. *J. Biol. Chem.* **259**:7622–7629 (1984).
8. Hausdorff, W. P., R. D. Sekura, G. Aguilera, and K. J. Catt. Control of aldosterone production by angiotensin II is mediated by two guanine nucleotide regulatory proteins. *Endocrinology* **120**:1668–1678 (1987).
9. Exton, J. H. Mechanism of action of calcium-mobilizing agonists: some variations on a young theme. *FASEB J.* **2**:2670–2676 (1988).
10. Pobiner, B. F., E. L. Hewlett, and J. C. Garrison. Role of N_i in coupling angiotensin II receptors to inhibition of adenylate cyclase in hepatocytes. *J. Biol. Chem.* **260**:16200–16209 (1985).
11. Marie, J., and S. Jard. Angiotensin II inhibits adenylate cyclase from adrenal cortex glomerulosa zone. *FEBS Lett.* **159**:97–100 (1983).
12. Hescheler, J., W. Rosenthal, K. D. Hinsch, M. Wulfern, W. Trautwein, and G. Schultz. Angiotensin II-induced stimulation of voltage-dependent Ca^{2+} currents in an adrenal cortical cell line. *EMBO J.* **7**:619–624 (1988).
13. Kojima, I., H. Shibata, and E. Ogata. Pertussis toxin blocks angiotensin II-induced calcium influx but not inositol trisphosphate production in adrenal glomerulosa cell. *FEBS Lett.* **204**:347–351 (1986).
14. Whitebread, S., M. Mele, B. Kamber, and M. De Gasparo. Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **163**:284–291 (1989).
15. Chiu, A. T., W. F. Herblin, D. E. McCall, R. J. Ardecky, D. J. Carini, J. V. Duncia, L. J. Pease, P. C. Wong, R. R. Wexler, A. L. Johnson, and P. B. M. W. M. Timmermans. Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **165**: 196–203 (1989).
16. Chang, R. S. L., and V. J. Lotti. Two distinct angiotensin II receptor binding sites in rat adrenal revealed by new selective nonpeptide ligands. *Mol. Pharmacol.* **29**:347–351 (1990).
17. Giroud, C. J. P., J. Stachenko, and E. H. Venning. Secretion of aldosterone by the zona glomerulosa of the rat adrenal gland incubated *in vitro*. *Proc. Soc. Exp. Biol. Med.* **92**: 154–158 (1956).
18. Guillemette, G., A. J. Baukal, T. Balla, and K. J. Catt. Angiotensin-induced formation and metabolism of inositol polyphosphates in bovine adrenal glomerulosa cells. *Biochem. Biophys. Res. Commun.* **142**: 15–22 (1987).
19. Sala, G. B., K. Hayashi, K. J. Catt, and M. L. Dufau. Adrenocorticotropin action in isolated adrenal cells: the intermediate role of cyclic AMP in stimulation of corticosterone synthesis. *J. Biol. Chem.* **254**:3861–3865 (1979).
20. Guardabasso, V., D. Rodbard, and P. J. Munson. A model-free approach to estimation of relative potency in dose-response curve analysis. *Am. J. Physiol.* **252**:E357–E364 (1987).
21. Balla, T., W. P. Hausdorff, A. J. Baukal, and K. J. Catt. Inositol polyphosphate production and regulation of cytosolic calcium during the biphasic activation of adrenal glomerulosa cells by angiotensin II. *Arch. Biochem. Biophys.* **270**:398–403 (1989).
22. Balla, T., A. Baukal, L. Hunyady, and K. J. Catt. Agonist-induced regulation of inositol tetrakisphosphate isomers and inositol pentakisphosphate in adrenal glomerulosa cells. *J. Biol. Chem.* **264**:13605–13611 (1989).
23. Chiu, A. T., D. E. McCall, T. Nguyen, D. J. Carini, J. V. Duncia, W. F. Herblin, R. T. Uyeda, P. C. Wong, R. R. Wexler, A. L. Johnson, and P. B. M. W. M. Timmermans. Discrimination of angiotensin II receptors subtypes by dithiothreitol. *Eur. J. Pharmacol.* **170**:117–118 (1989).
24. Catt, K. J., F. A. Mendelsohn, M. A. Millan, and G. Aguilera. The role of angiotensin II receptors in vascular regulation. *J. Cardiovasc. Pharmacol.* **6**:S575–S586 (1984).
25. Wong, P. C., S. D. Hart, A. M. Zaspel, A. T. Chiu, J. Ardecky, R. D. Smith, and P. B. M. W. M. Timmermans. Functional studies of nonpeptide angiotensin II receptor subtype-specific ligands. Dup 753 (AII-1) and PD 123177 (AII-2). *J. Pharmcol. Exp. Ther.* **255**:584–592 (1990).
26. Woodcock, E. A., and C. I. Johnston. Inhibition of adenylate cyclase in rat adrenal glomerulosa cells by angiotensin II. *Endocrinology* **115**:337–341 (1984).
27. Baukal, A. J., T. Balla, L. Hunyady, W. Hausdorff, G. Guillemette, and K. J. Catt. Angiotensin II and guanine nucleotides stimulate formation of inositol 1,4,5-trisphosphate and its metabolites in permeabilized adrenal glomerulosa cells. *J. Biol. Chem.* **263**:6087–6092 (1988).
28. Carson, M. C., C. M. Harper, A. J. Baukal, G. Aguilera, and K. J. Catt. Physicochemical characterization of photoaffinity-labeled angiotensin II receptors. *Mol. Endocrinol.* **1**:147–153 (1987).
29. Ji, H., K. Sandberg, and K. J. Catt. Novel angiotensin II antagonists distinguish amphibian from mammalian angiotensin II receptors expressed in *Xenopus laevis* oocytes. *Mol. Pharmacol.* **39**:122–123 (1991).
30. Dudley, D. T., R. L. Panek, T. C. Major, G. H. Lu, R. F. Bruns, B. A. Klinkefus, J. C. Hodges, and R. E. Weishaar. Subclasses of angiotensin II binding sites and their functional significance. *Mol. Pharmacol.* **38**:370–377 (1990).
31. Speth, R., and K. H. Kim. Discrimination of two angiotensin II receptor subtypes with a selective agonist analogue of angiotensin II, *p*-aminophenylalanine^6 angiotensin II. *Biochem. Biophys. Res. Commun.* **169**:997–1006 (1990).

Send reprint requests to: Kevin J. Catt, Endocrinology and Reproduction Research Branch, NICHHD, NIH, Bldg. 10, Room B1/L-400, Bethesda, MD 20892.