0026-895X/90/030370-08$02.00/0

MOLECULAR PHARMACOLOGY, 38:370-377

Subclasses of Angiotensin II Binding Sites and Their Functional Significance

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Received January 22, 1990; Accepted June 21, 1990

SUMMARY

Specific binding sites for angiotensin II were identified in the rabbit adrenal gland and uterus and in the rat liver and were divided into two subclasses based on inhibition by nonpeptide ligands. Peptide ligands affected binding equally in all three tissues. However, the nonpeptide antagonists Dup 753 and Exp 6803 blocked angiotensin II binding to adrenal and liver homogenates at nanomolar concentrations but exerted only a minimal effect on binding to uterine homogenates. The nonpeptide PD 123319 potently blocked angiotensin II binding to uterine homogenates but had no effect on adrenal or liver homogenates at concentrations up to 10 μM. Further analysis of angiotensin II binding in uterus showed that both sites are present, with the PD 123319-sensitive site predominating. Additionally, the nonhydrolyzable GTP analogue 5′-guanylyl-imidodiphosphate was able to modulate binding to liver and to the Dup 753-sensitive site in uterus but not that to the PD 123319-sensitive site. Saralasin and the nonpeptide antagonists Dup 753 and Exp 6803 blocked angiotensin II-stimulated accumulation of inositol phosphates in cultured Clone 9 cells and also relaxed aortic rings previously contracted with angiotensin II. In contrast, PD 123319 had no effect on either angiotensin II-stimulated inositol phosphate accumulation or vasoconstriction. Saralasin and Exp 6803, but not PD 123319, lowered blood pressure in renal hypertensive rats following intravenous administration. These results suggest the existence of two subclasses of angiotensin II binding sites, which differ in their tissue distribution and affinity for the nonpeptide ligands Dup 763, Exp 6803, and PD 123319. Although no functional role for the PD 123319-sensitive subclass has yet been identified, the Dup 753/Exp 6803-sensitive subclass plays an important role in mediating inositol phosphate metabolism, vascular contractile activity, and blood pressure regulation.

The role of the renin-angiotensin system in regulating blood pressure is well established (1–3). Available evidence suggests that this involvement is mediated by the concerted effects of the octapeptide angiotensin II on several key processes, including vascular muscle contractility, salt and water excretion, and neurotransmitter release (4). In addition, angiotensin II is a growth factor and has been linked to the development of vascular and cardiac muscle hypertrophy (5, 6). The role of these latter events in contributing to the hypertensive effect of angiotensin II remains unclear.

Most studies linking angiotensin II to the regulation of blood pressure have relied on plasma measurements of various components of the renin-angiotensin system, e.g., plasma renin (7, 8), or on pharmacological agents that either inhibit angiotensin II synthesis or block the binding of angiotensin II to its receptor, e.g., ACE inhibitors and peptide receptor antagonists, respectively (1, 3, 9). The use of plasma markers to characterize the activity of the renin-angiotensin system has limitations, because a disassociation between changes in the plasma levels of these markers and changes in blood pressure has been reported by several investigators (10–12). Use of renin or ACE inhibitors may also have limitations, because reports exist of "non-renin-" and "non-ACE-" dependent synthesis of angiotensin II (13, 14). Finally, peptide receptor antagonists such as saralasin often produce varying degrees of agonist activity (15), making interpretation of results obtained with these compounds difficult.

The recent identification of nonpeptide angiotensin II receptor antagonists that lack partial agonist activity provides the most direct approach to study of the involvement of the renin-angiotensin system in blood pressure regulation (16–18). Furthermore, the nonpeptide structures have revealed the presence of multiple angiotensin II binding sites (19–21), raising the possibility that angiotensin II may have additional physiological effects that are unappreciated at present.

In characterizing the *in vitro* and *in vivo* effect of various angiotensin II antagonists, we also identified two subclasses of

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ABBREVIATIONS: ACE, angiotensin-converting enzyme; $G_{pp}(NH)_p$, 5′-guanylyl-imidodiphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin, DMSO, dimethyl sulfoxide; G protein, guanine nucleotide-binding protein.

binding sites for angiotensin II, which differed in their sensitivity to nonpeptide antagonists such as Exp 6803 and PD 123319 but not to peptide antagonists such as saralasin. The purpose of the present study was to evaluate differences in tissue distribution for the two subclasses and to characterize the involvement of each in regulating functional activities such as polyphosphoinositide turnover, *in vitro* contractility, and blood pressure.

Experimental Procedures

Materials. [*tyrosyl*-3,5-^{3}H(N)]Angiotensin II (specific activity 59.9 Ci/mmol) ^{125}I-angiotensin II (2200 Ci/mmol), and ^{125}[Sar^{1}Ile^8]angiotensin II (2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [^{3}H]Inositol (23 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). $G_{pp}(NH)_p$ was obtained from CalBioChem (San Diego, CA). Angiotensin peptides were obtained from either Bachem Fine Chemicals (Torrance, CA), Peninsula Laboratories (Belmont, CA), or Sigma Chemical Co. (St. Louis, MO). PD 123319, Dup 753, and Exp 6803 were prepared according to methods described in the patent literature (22–24). The structures of these latter three compounds and the peptide antagonists saralasin ([Sar^1,Val^5,Ala^8]-angiotensin II) are illustrated in Fig. 1.

Angiotensin II binding assay. Crude membranes were prepared from rat liver, rabbit uterus, or rabbit adrenals (adrenals were purchased directly from Pel-Freeze, Rogers, AK). Each tissue was homogenized in 10 volumes of ice-cold 10 mM HEPES buffer (adjusted to pH 7.4 with Tris base), containing 10 μM leupeptin, bestatin, pepstatin A, and captopril and 100 μM phenylmethylsulfonyl fluoride, for 20 sec using a Brinkmann Polytron PT-10 at setting 7. The homogenates were centrifuged at 50,000 × *g* for 10 min, and the supernatant was discarded. The resulting pellets were washed once in ice-cold HEPES buffer, centrifuged as above, and resuspended in 5 volumes of HEPES buffer. Membranes were either used immediately or stored in aliquots at −70° for up to 1 month.

Binding of [^{3}H]angiotensin II to membranes was conducted in a final volume of 1 ml of HEPES buffer (supplemented with protease inhibitors as above, 0.2% BSA, and 10 mM $MgCl_2$), containing 10 mg of original tissue weight of homogenate, 0.5 nM [^{3}H]angiotensin II, and test compound. In some experiments, 25 pM ^{125}I-angiotensin II or ^{125}I-[Sar^{1}Ile^8]-angiotensin II was used as the radioligand. If necessary, test compounds were dissolved in DMSO. Control incubations received an equal amount of DMSO, which had no effect on binding. Samples were incubated at 25° for 60 min and binding was terminated by filtration through Whatman GF/B glass fiber filter sheets (presoaked in 50 mM Tris buffer, pH 7.7, containing 0.2% BSA and 100 μM bacitracin), using a Brandel 48R cell harvester. Filters were washed three times with 4 ml of Tris buffer and then transferred to scintillation vials. Formula 963 scintillation fluid (DuPont) was added and radioactivity was determined in a liquid scintillation counter. Nonspecific binding was defined as radioactivity retained on the filters in the presence of 10 μM saralasin, and specific binding was defined as total binding minus nonspecific binding. IC_{50} values were calculated by weighted nonlinear regression curve-fitting to the mass-action equation (25).

Cell culture and inositol phosphate accumulation assay. Clone 9 cells, derived from rat liver, were originally obtained from the American Type Culture Collection (Rockville, MD) and were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum. For determination of [^{3}H]inositol phosphates, cells were seeded into 12-well plates and labeled by addition of 1 μCi/ml [^{3}H]inositol to the growth medium. After labeling for 48 hr, cells were washed and then incubated with minimal essential medium supplemented with 20 mM HEPES, 10 mM LiCl, and 0.1% BSA. Test compounds were incubated with the cells for 60 min at 37°. To terminate the incubation, the medium was aspirated and replaced with 0.5 ml of ice-cold 5% trichloroacetic acid. Total [^{3}H]inositol phosphates were analyzed by a method similar to the procedure of Downes and Michell (26), by applying the

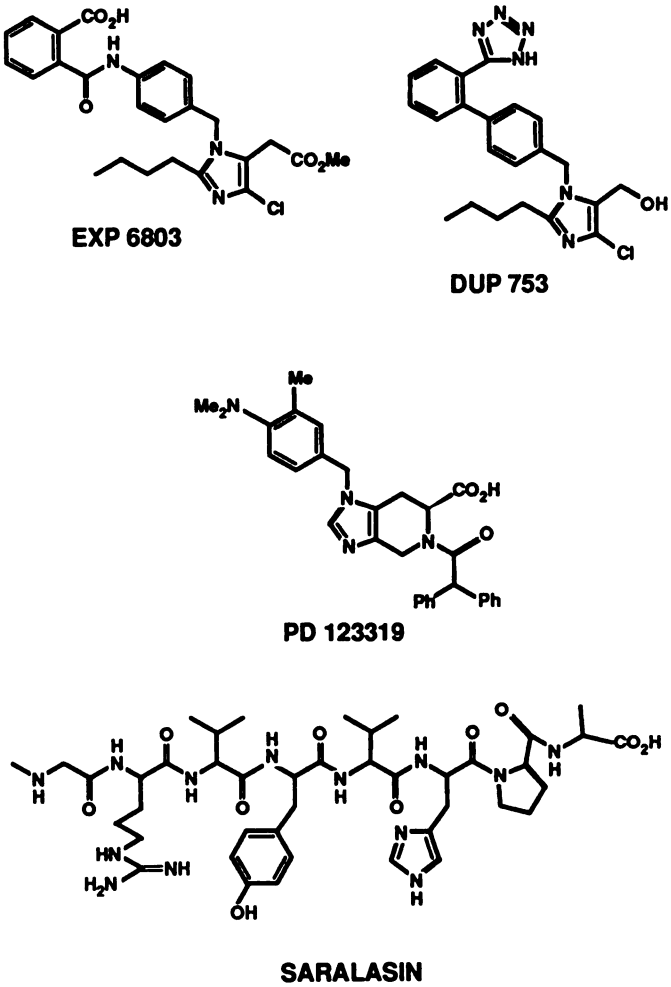

Fig. 1. Chemical structures of the nonpeptide angiotensin II binding site antagonists Dup 753, Exp 6803, and PD 123319 and the peptide antagonist saralasin ([Sar^1,Val^5,Ala^8]-angiotensin II). Dup 753 and Exp 6803 were discovered at E.I. du Pont de Nemours & Co. (Wilmington, DE). PD 123319 was discovered at Parke-Davis Pharmaceutical Research/Warner-Lambert Co. (Ann Arbor, MI).

trichloroacetic acid extract directly to Dowex anion exchange columns. [^{3}H]Inositol was removed by washing with 10 ml of 5 mM inositol, and total [^{3}H]inositol phosphates were eluted with 4 ml of 1 M ammonium formate in 0.1 M formic acid.

***In vitro* contractility in isolated rabbit aorta.** Aortas were removed from adult New Zealand white male rabbits (2–4 kg; Hazelton Research Products, Inc., Denver, PA) following cervical dislocation, and 5-mm-wide rings were taken from the descending thoracic region of the aorta. These were mounted in 20-ml tissue baths containing Krebs bicarbonate solution of the following composition (in mM): NaCl, 118.2; KCl, 4.6; KH_2PO_4, 1.2; $NaHCO_3$, 24.8; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $CaNa_2EDTA$, 0.026; dextrose, 10.0; pH 7.4. The buffer was maintained at 37° and gassed continuously with 5% CO_2 in oxygen. The segments were connected to isometric force transducers, and resting tension was adjusted to 4.0 g. After a 90-min equilibration period, the rings were contracted with 122 mM KCl and then washed with fresh Krebs buffer. The segments were then stimulated to contract with 10 nM angiotensin II, which produced 70% of the maximum response to angiotensin II. After washing with fresh Krebs buffer, the rings were stimulated a second time with 10 nM angiotensin II in order to establish a control response and to demonstrate the absence of tachyphylaxis. After washing for 30 min, saralasin, Dup 753, Exp 6803, or PD 123319 was then added to the bath, and 10 min later the rings were again challenged with angiotensin II. After the maximum response to angiotensin II was

obtained, the rings were washed and allowed to reequilibrate for 30 min. Increasing concentrations of the drugs were then added and the response to angiotensin II was repeated until the maximum antagonist response was obtained or the limit of solubility was reached. The vehicle for these studies, DMSO, inhibited angiotensin II-dependent contraction less than 10%.

***In vitro* contractility in isolated rabbit uterus.** Uterine contractile responses were evaluated using the technique of Scanlon and Moore (27). Adult New Zealand white female rabbits (3–4 kg; Hazelton Research Products, Inc.) were used for these studies. Following cervical dislocation, the uterus was exposed and both uterine horns were removed, cut into 1-cm segments, and suspended in 20-ml tissue baths containing DeJalons solution of the following composition (in mM): NaCl, 150; KCl, 5.6; $CaCl_2$, 0.18; $NaHCO_3$, 1.8, dextrose, 1.4; pH 7.4. The buffer was maintained at 29° and gassed continuously with 5% CO_2 in oxygen. A protocol similar to that described for aortic rings was used to measure uterine responses to angiotensin II at a resting tension of 1 g. The response to angiotensin peptides in uterine smooth muscle was characterized by oscillatory contractile activity and small variations in sensitivity. Angiotensin II antagonism was measured by determination of percentage of inhibition of the peak angiotensin II constrictor response (reduction in the height of the largest spike in a 10-min period). The concentration of angiotensin II used for eliciting a response was either 30 or 70 nM, depending on the responsiveness of the tissue to angiotensin II on the day of each experiment.

***In vivo* blood pressure studies.** Two-kidney, one-clip, renal hypertensive rats were used for these studies. Hypertension was produced by partially occluding the left renal arteries of male Sprague-Dawley rats with a silver clip of 0.2-mm gap (28). Eight to 10 weeks later, indwelling catheters were implanted for direct measurement of arterial blood pressure and heart rate and for intravenous administration of drugs. All experiments were conducted in conscious unrestrained rats. Saralasin, Exp 6803, and PD 123319 were administered as single intravenous bolus injections in increasing doses to renal hypertensive and normotensive rats. Blood pressure and heart rate were monitored continuously. Predrug control blood pressure and heart rate were obtained by averaging two 15-min intervals before compound administration.

Results

Angiotensin II binding. [^{3}H]Angiotensin II bound to membranes from rat liver, rabbit adrenal, and rabbit uterus in a specific saturable manner. Scatchard analysis (data not shown) revealed similar affinities for angiotensin II in all three tissues (K_d = 2.6–5.5 nM). In competition experiments, a number of angiotensin II peptide analogues, including saralasin, inhibited binding of [^{3}H]angiotensin II to each tissue. The inhibitory potency for these peptide analogues was comparable in the different tissues, suggesting similar binding sites for angiotensin II (Fig. 2 and Table 1).

The nonpeptide antagonists Dup 753 and Exp 6803 also inhibited binding of [^{3}H]angiotensin II to membranes from rat liver and rabbit adrenal. However, Dup 753 exhibited biphasic binding to rabbit uterus membranes (Fig. 2C), and both of these nonpeptide compounds were much less potent in inhibiting binding to this tissue, as compared with liver and adrenal. In contrast to Dup 753 and Exp 6803, PD 123319 potently inhibited binding of [^{3}H]angiotensin II to rabbit uterus membranes, while exerting little inhibitory effect on binding to rat liver or rabbit adrenal membranes (Fig. 2 and Table 1). Although PD 123319 was very potent at blocking [^{3}H]angiotensin II binding in rabbit uterus (IC_{50} = 21.2 nM), it only displaced about 70% of the radioactivity. These observations suggested that [^{3}H]-angiotensin II may be interacting with multiple sites in rabbit

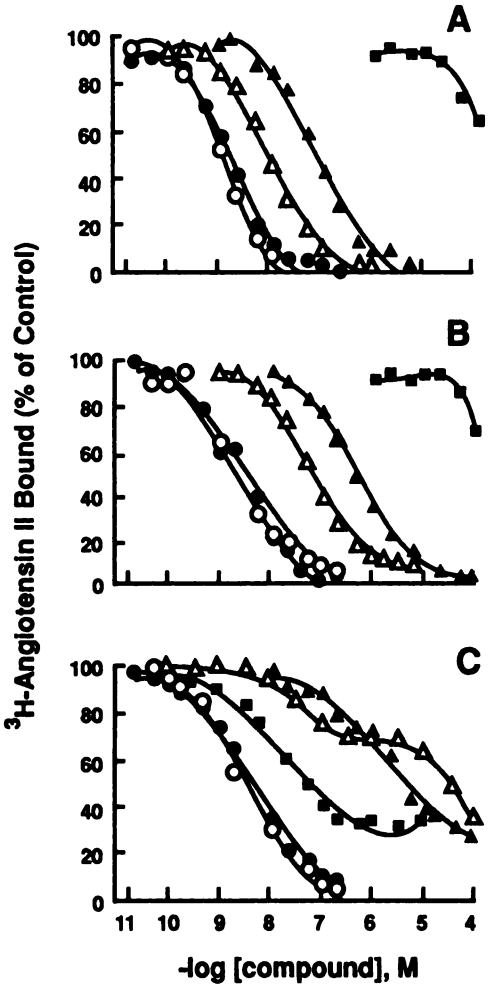

Fig. 2. Effect of increasing concentrations of angiotensin II (○), saralasin (●), Dup 753 (△), Exp 6803 (▲), or PD 123319 (■) on specific binding of [^{3}H]angiotensin II to membranes isolated from rat liver (A), rabbit adrenal (B), and rabbit uterus (C). Each *point* represents the mean of triplicate determinations. Binding to the membranes was determined as described in Experimental Procedures.

uterus and that PD 123319 and Dup 753 may be recognizing different sites.

To further explore the possibility of multiple binding sites in rabbit uterus, tissue was incubated with ^{125}I-angiotensin II and dose-displacement curves of PD 123319 and Dup 753 were obtained. Fig. 3A shows that Dup 753 generates a biphasic displacement curve, similar to that obtained when [^{3}H]angiotensin II is used. In the presence of 1 μM PD 123319, about 40% of the specific binding is blocked and the curve loses its biphasic nature, leaving an apparent high affinity binding site for Dup 753. Furthermore, this high affinity site corresponds to the Dup 753-sensitive site seen in rat liver and rabbit adrenal (Fig. 2 and Table 1).

Fig. 3B shows a dose inhibition of ^{125}I-angiotensin II binding by PD 123319 in rabbit uterus. This curve shows one inflection, but PD 123319 at up to 10^{-4} M fails to completely displace the radioactive tracer. In the presence of 1 μM Dup 753, the curve retains its shape and is shifted downward. Interestingly, the combination of 1 μM Dup 753 and PD 123319 can completely displace the radioactive tracer. These results show that rabbit uterus contains two types of binding sites. One site is sensitive to PD 123319 and to high concentrations of Dup 753. The other

TABLE 1

Values for angiotensin reference agents in binding and functional assays

Values for binding assays are concentrations required to displace 50% of the maximum displaceable [^{3}H]angiotensin II. Functional assays are accumulation of inositol phosphates (Inos PO_4) in Clone 9 cells and contraction of rabbit aortic or uterine smooth muscle. AI, AII, and AIII, angiotensins I, II, and III.

Agonists	EC_{50}					
	Binding assays			Functional assays		
	Rat liver	Rabbit adrenal	Rabbit uterus	Inos PO_4 accumulation	Aorta contraction	Uterus contraction
	nM					
[Sar^1]-AII	2.03	2.93	1.64	0.30	1.4	NT[a]
AII	1.02	1.51	2.50	0.85	2.5	3.5
AIII	5.57	10.83	1.74	4.12	32	24
AI	300	579	116	232	10,000	33
Antagonists	IC_{50}					
	Binding assays			Functional assays		
	Rat liver	Rabbit adrenal	Rabbit uterus	Inos PO_4 accumulation	Aorta contraction	Uterus contraction
	nM					
[Sar^1,Val^5,Ala^8]-AII	1.69	3.63	1.32	2.66	7.6	[89%][b]
[Sar^1,Leu^8]-AII	3.22	5.72	2.56	0.51	1.0	NT
[Sar^1,Thr^8]-AII	3.05	6.91	0.92	2.07	3.3	NT
[Sar^1,Ala^8]-AII	3.10	6.52	1.61	2.84	1.8	NT
[Sar^1,Ile^8]-AII	7.80	14.9	3.48	3.12	0.7	NT
[des-Asp^1,Ile^8]-AII	4.45	7.59	0.99	4.52	62	NT
Dup 753	7.52	38.5	35.9, >10,000[c]	19.0	85	[59%][b]
Exp 6803	44.9	366	2,655	340	765	NT
PD 123319	>10,000	>10,000	21.2	>10,000	>10,000	[0%][b]

[a] NT, not tested.
[b] Percentage of inhibition of the response to angiotensin II at 3 nM test compound.
[c] Values for Dup 753 were obtained after fit to a two-site model (25).

site is recognized by nanomolar levels of Dup 753 and is not recognized by PD 123319 at concentrations up to 10^{-4} M.

Angiotensin II receptors have been shown to be modulated by G proteins (29–31). To determine whether the binding sites delineated by Dup 753 and PD 123319 might be affected in a similar manner, binding assays were conducted using the radioactive antagonist ^{125}I-[Sar^1,Ile^8]-angiotensin II. Agonist displacement curves were then obtained using unlabeled angiotensin II in the presence and absence of the nonhydrolyzable guanine nucleotide analogue $G_{pp}(NH)_p$. Fig. 4A shows that $G_{pp}(NH)_p$ was able to shift the binding curve obtained using rat liver membranes. In Fig. 4B, using rabbit uterus membranes in which 1 μM PD 123319 has been included to eliminate binding to these sites, $G_{pp}(NH)_p$ was also able to shift the binding curve. However, in Fig. 4C, in which 1 μM Dup 753 has been included to eliminate binding to the Dup 753 sites, $G_{pp}(NH)_p$ was unable to influence the binding curve. These data suggest that the angiotensin II binding sites recognized by Dup 753 are coupled to an effector through G proteins, whereas sites recognized by PD 123319 are not.

In order to examine potential coupling mechanisms and functional activities of the angiotensin II binding sites, we utilized cultured cells and several *in vitro* and *in vivo* preparations.

Stimulated turnover of inositol lipids in Clone 9 cells. Rat liver Clone 9 cells were found to express angiotensin II binding sites that recognize Dup 753 but not PD 123319 (data not shown). Treatment of [^{3}H]inositol-labeled Clone 9 cells with angiotensin II resulted in a large increase in the level of total [^{3}H]inositol phosphates. This response was time dependent, reaching a plateau by about 30 min (data not shown). Examination of the concentration dependence showed that angiotensin II yielded a sigmoidal curve, generating a maximal response at 100 nM with an EC_{50} value of 0.9 nM (Fig. 5). Angiotensin I, [Sar^1]-angiotensin II, and angiotensin III also generated agonist responses in Clone 9 cells (Fig. 5, Table 1). [Sar^1]-angiotensin II was more potent than angiotensin II, with an EC_{50} value of 0.3 nM, whereas angiotensin III was less potent, with an EC_{50} value of 4 nM. Angiotensin I was much less potent, with an EC_{50} of 232 nM. In addition, angiotensin I was unable to elucidate as large a response as the other agonists.

In order to evaluate antagonist effects on [^{3}H]inositol phosphate accumulation, cells were stimulated with 1 nM angiotensin II in the presence of varying concentrations of different antagonists. Fig. 6 shows the results for saralasin, Dup 753, Exp 6803, and PD 123319. Saralasin was a potent antagonist of angiotensin II-stimulated inositol phosphate accumulation in Clone 9 cells, yielding an IC_{50} value of 2.7 nM. Two of the nonpeptides were also potent antagonists; IC_{50} values of 19 and 340 nM were obtained for Dup 753 and Exp 6803, respectively. Additionally, saralasin, as well as both Dup 753 and Exp 6803, produced a parallel shift to the right of the dose response to angiotensin II, suggesting a competitive interaction with the angiotensin II receptor (data not shown). In agreement with lack of an effect on binding of angiotensin II to these cells, PD 123319 had only a minimal effect on [^{3}H]inositol phosphate accumulation in Clone 9 cells (Fig. 6 and Table 1).

***In vitro* contractility.** Fig. 7 shows the effect of saralasin, Dup 753, Exp 6803, and PD 123319 on the contractile response to 10 nM angiotensin II in rabbit isolated aorta. Saralasin, Dup 753, and Exp 6803 all caused concentration-dependent inhibition of the response. The order of potency for these antagonistic effects was saralasin > Dup 753 > Exp 6803, the same as obtained for inhibition of [^{3}H]inositol phosphate accumulation

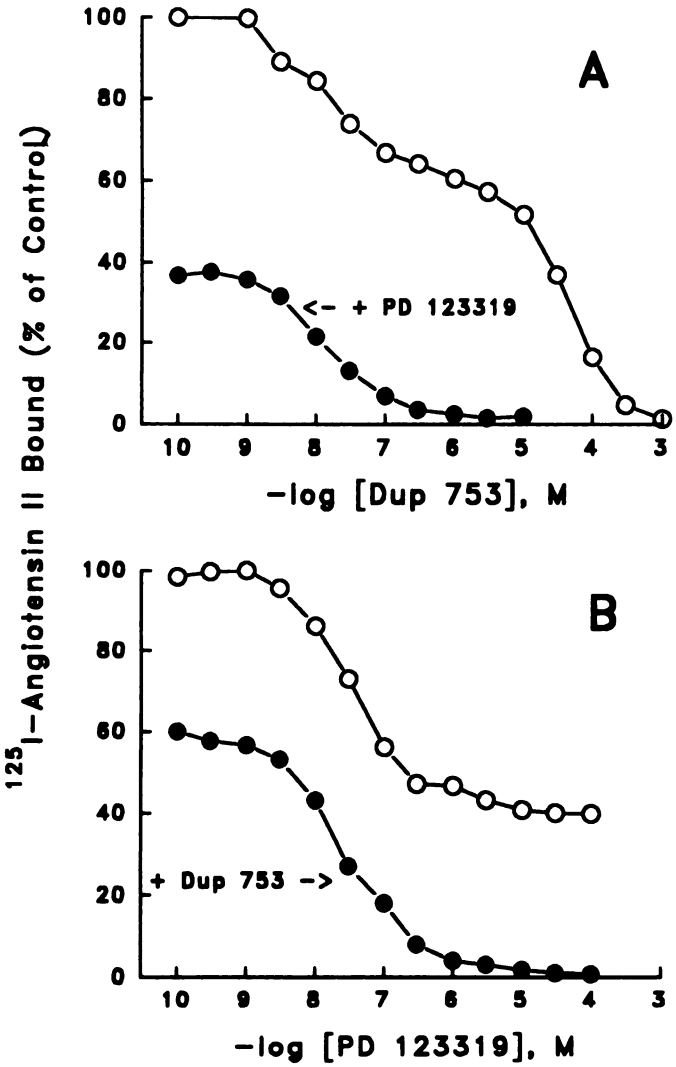

Fig. 3. Effect of PD 123319 and Dup 753 on ^{125}I-angiotensin II binding to uterus membranes. A, ^{125}I-Angiotensin II was displaced by increasing concentrations of Dup 753, either alone (○) or in the presence of 1 μM PD 123319 (●). B, ^{125}I-Angiotensin II was displaced by increasing concentrations of PD 123319, either alone (○) or in the presence of 1 μM Dup 753 (●). Shown is a representative example of three separate experiments.

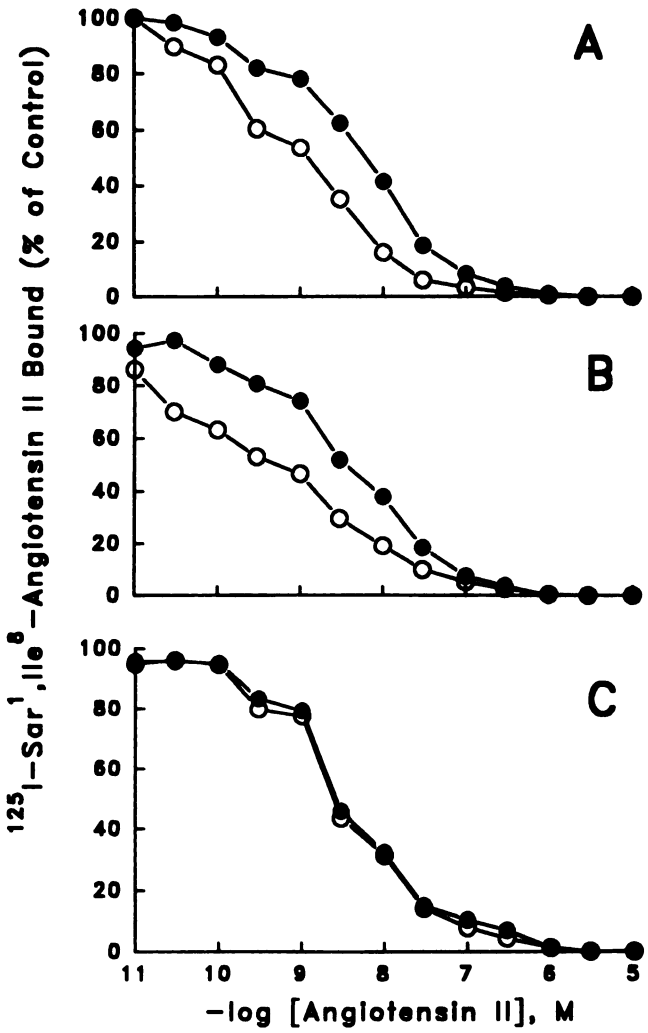

Fig. 4. Effect of $G_{pp}(NH)_p$ on angiotensin II binding to Dup 753- and PD 123319-sensitive binding sites. Binding was performed as described in Experimental Procedures, with the inclusion of 120 mM NaCl in the assay buffer. ^{125}I-[Sar^{1},Ile^{8}]angiotensin II was displaced by increasing concentrations of unlabeled angiotensin II in the presence (●) or absence (○) of 100 μM $G_{pp}(NH)_p$. A, Binding using rat liver homogenates. B, Binding in rabbit uterine homogenates in the presence of 1 μM PD 123319 (measuring the effect on Dup 753-sensitive sites). C, Binding in rabbit uterine homogenates in the presence of 1 μM Dup 753 (measuring the effect on PD 123319-sensitive sites). Shown is a representative example of two separate experiments.

in Clone 9 cells. In contrast to saralasin, Dup 753, and Exp 6803, PD 123319 exerted no effect on angiotensin II-induced contractions (Fig. 7 and Table 1). The antagonistic activity of saralasin, Dup 753, and Exp 6803 was selective for angiotensin II, because none of these compounds exerted a significant effect on norepinephrine-induced contractions (<5% inhibition at 1 μM; data not shown).

Results for *in vitro* contractility in the rabbit isolated uterus were similar to those in the isolated rabbit aorta. At a concentration of 3 nM, saralasin and Dup 753 inhibited the constrictor response to angiotensin II by 89 and 59%, respectively (Fig. 8 and Table 1). PD 123319, however, had no effect on the response to angiotensin II at a concentration of 1 μM.

***In vivo* antihypertensive results.** The antihypertensive effects of saralasin, Exp 6803, and PD 123319 were evaluated in renal hypertensive rats. Both saralasin (0.003–0.3 mg/kg) and Exp 6803 (0.1–30 mg/kg) produced dose-related reductions in mean arterial blood pressure when administered as an intravenous bolus injection (Fig. 9, A and B). Saralasin (0.3 mg/kg) produced an initial rapid reduction in blood pressure, with a maximal decrease of 24 mm Hg occurring within 3 min (Fig. 9A). Blood pressure returned to control levels within 30 min following the initial challenge. Similarly, Exp 6803 caused an initial rapid reduction in blood pressure, with a maximal decrease of 30 mm Hg at a dose of 3 mg/kg, intravenously (Fig. 9B). In contrast to saralasin, the duration of the antihypertensive response to Exp 6803 at 30mg/kg was more than 30 min. When administered at doses ranging from 0.3 to 30 mg/kg, PD 123319 produced no reduction in mean blood pressure (Fig. 9C). Additionally, neither saralasin nor Exp 6803 had any effect on blood pressure in normotensive rats at doses up to 0.3 mg/kg (for saralasin) and 30 mg/kg (for Exp 6803).

Discussion

The octapeptide angiotensin II mediates a variety of responses in several tissues, including contraction of vascular

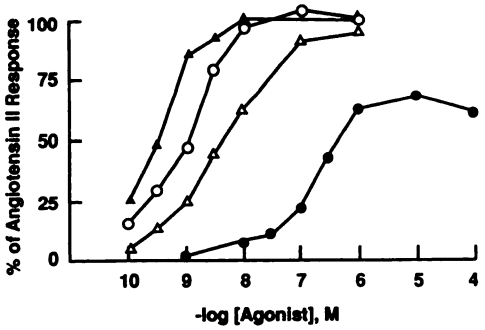

Fig. 5. Stimulatory effects of increasing concentrations of angiotensin I (●), angiotensin II (○), angiotensin III (△), and [Sar¹]-angiotensin II (▲) on inositol phosphate accumulation in Clone 9 cells. Inositol phosphate levels were determined as described in Experimental Procedures. Each *point* represents the mean of triplicate determinations; *error bars* were omitted for clarity but were typically smaller than the *symbol*.

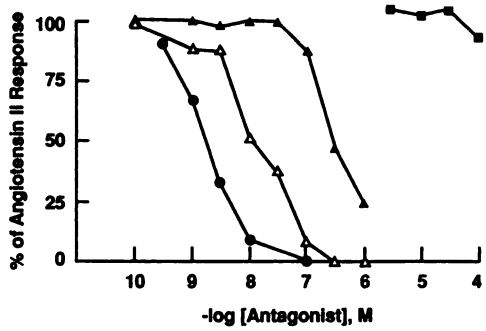

Fig. 6. Inhibitory effects of increasing concentrations of saralasin (●), Dup 753 (△), Exp 6803 (▲), or PD 123319 (■) on inositol phosphate accumulation in Clone 9 cells stimulated with 1 nM angiotensin II. Each *point* represents the mean of triplicate determinations; *error bars* were omitted for clarity but were typically smaller than the symbol.

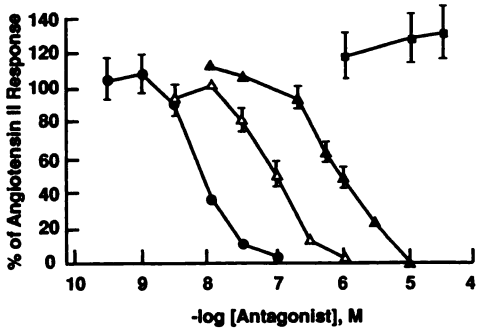

Fig. 7. Inhibitory effect of increasing concentrations of saralasin (●), Dup 753 (△), Exp 6803 (▲), or PD 123319 (■) on the contractile response to angiotensin II in isolated rabbit aortic rings. The procedures used to measure changes in aortic contractility are described in Experimental Procedures. Each point represents the mean ± standard error of four separate determinations.

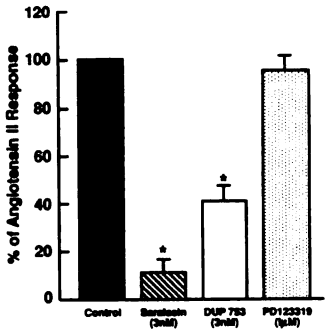

Fig. 8. Inhibitory effect of 3 nM saralasin, 3 nM Dup 753, or 1 μM PD 123319 on the constrictor response to angiotensin II in isolated rabbit uterine rings. The procedures used to measure changes in uterine contractility are described in Experimental Procedures. Each *bar* represents the mean ± standard error of four separate determinations.

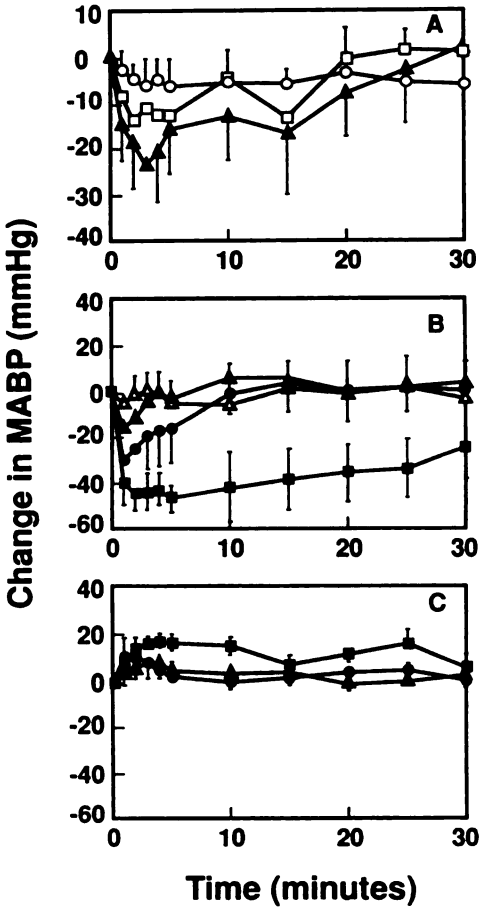

Fig. 9. Blood pressure responses to increasing doses of saralasin (A), Exp 6803 (B), and PD 123319 (C) administered intravenously to renal hypertensive rats. Doses (mg/kg) were 0.003 (○), 0.03 (□), and 0.3 (▲) for saralasin, 0.1 (△), 1.0 (▲), 3.0 (●), and 30 (■) for Exp 6803, and 0.3 (▲), 3.0 (●), and 30 (■) for PD 123319. The procedures used to induce renal hypertension and measure blood pressure *in vivo* are described in Experimental Procedures. Each *point* represents the mean ± standard error of three to five hypertensive rats. *MABP*, mean arterial blood pressure.

smooth muscle, excretion of salt and water from kidney, release of prolactin from pituitary, and stimulation of aldosterone secretion from the adrenal gland (4, 32–34). There is further evidence suggesting that angiotensin II is involved in regulating cell growth in both cardiac and vascular tissue (5, 6). Although binding sites for angiotensin II have been identified in many target organs and cells (35–38), little is known regarding the structure, function, and regulation of these binding sites. Initial studies indicated that the angiotensin II peptide analogue saralasin produced comparable antagonistic effects on all angiotensin II-dependent responses (15), suggesting that the responses were mediated by the same receptor. Goghari *et al.* (39) subsequently divided the peptide-type angiotensin II antagonists into two groups, based on their ability to bind competitively or noncompetitively to the receptor and the reversibility of the antagonist activity. Differences have also been reported

in the agonist activity of various angiotensin peptides, with binding and functional studies suggesting that in the brain angiotensin III is the preferred agonist (40) and studies in peripheral tissues indicating that angiotensin II has greater activity (35, 38). Whether these differences are due to separate receptors for angiotensin II and angiotensin III or to subclasses of a single angiotensin II receptor remains to be established.

The recent description of nonpeptide angiotensin II antagonists has also led to differentiation of angiotensin II binding sites in several tissues. Whitebread *et al.* (19) found two disparate angiotensin II binding sites in rat and human adrenal and uterus, based on sensitivity to CGP 42112A (termed type A sites) or Ex 89 (termed type B sites). It should be noted that Ex 89 is the same structure as Dup 753. Binding to the type B sites was also found to be abolished by the sulfhydryl reagent dithiothreitol, whereas dithiothreitol enhanced binding to the type A site. Similarly Chiu and co-workers (20) used Dup 753 and Exp 655 (a structural analogue of PD 123319) to distinguish angiotensin II binding sites in rat adrenal. These sites were termed type 1 (sensitive to Dup 753) and type 2 (sensitive to Exp 655). Recently, Chang and Lotti (21), using similar nonpeptide ligands, also showed separate angiotensin II binding sites in rat adrenal gland. These workers also noted that the type 1 site (recognized by Dup 753) is sensitive to dithiothreitol, whereas the type 2 site (recognized by the PD 123319 analogue WL-19) is not, and they further showed that angiotensin II-stimulated aldosterone release could be blocked by Dup 753 but not by type 2 binding site blockers.

In the present study, we similarly found two distinct binding sites for angiotensin II, based on differences in the displacement of radiolabeled angiotensin II by nonpeptide ligands. We further noted several functional effects of angiotensin II, which were clearly associated with one of the binding sites. These results lend considerable weight to the possibility of multiple angiotensin II receptor subtypes and strongly suggest that at least two subtypes exist that have differential tissue distribution and functional responses.

All the tissues examined in this study for angiotensin II binding activity showed approximately equal parameters for the variety of peptide ligands used. Conversely, the nonpeptide ligands showed quite different abilities to displace radiolabeled angiotensin II in rat liver and adrenal versus rabbit uterus. Dup 753 and Exp 6803 were able to effectively inhibit binding to liver and adrenal, whereas PD 123319 was ineffective. However, uterus binding was potently blocked by PD 123319, although this compound could not displace all of the added radioligand. Additionally, Dup 753 displayed a biphasic binding curve, suggestive of interaction at multiple binding sites. Further studies revealed two binding sites in uterus that were recognized by either Dup 753 or PD 123319. It was also apparent that binding to the uterine site recognized by Dup 753 could be modulated with the guanine nucleotide analogue $G_{pp}(NH)_p$, whereas the site recognized by PD 123319 was unaffected by this treatment. This raises the possibility that the sites recognized by Dup 753 are coupled to a functional response through G proteins, whereas the PD 123319-sensitive sites are not, and further serves to distinguish these disparate angiotensin II binding sites.

A number of functional responses were evaluated for their sensitivity to the peptide and nonpeptide ligands. Clone 9 cells were identified as a useful cultured cell model that expresses angiotensin II binding sites that are sensitive to Dup 753 but are insensitive to PD 123319 (data not shown). Further, in these cells, angiotensin II stimulates inositol phospholipid turnover in a manner similar to that in primary adrenal glomerulosa cultures (41, 42). Examination of a variety of angiotensin II peptide analogues for ability to stimulate accumulation of inositol phosphates showed an excellent pharmacological correlation with smooth muscle assays. Furthermore, both Dup 753 and Exp 6803 were able to block angiotensin II-stimulated turnover of inositol phospholipids in Clone 9 cells, whereas PD 123319 was ineffective. Additionally, Dup 753 and Exp 6803 were unable to block vasopressin or platelet-activating factor-induced accumulation of inositol phosphates in Clone 9 cells (data not shown), indicating that these nonpeptide ligands are interacting with the receptor and are not nonspecifically blocking transmission of the angiotensin II signal across the membrane. Moreover, other receptor systems that are coupled to turnover of inositol phospholipids are related to the rhodopsin family of receptors and appear to be activated through G proteins (43). Our binding and functional results indicate that the Dup 753/Exp 6803-sensitive site may be related to this family as well.

The nonpeptide ligands Dup 753 and Exp 6803 were also able to specifically block contraction of aortic and uterine smooth muscle induced by angiotensin II, whereas PD 123319 was ineffective. Likewise, Exp 6803 caused *in vivo* decreases in blood pressure, similar to effects noted with saralasin. PD 123319, in contrast, was unable to effect any changes upon measurements of blood pressure. Our functional measurements of inositol phospholipid turnover, smooth muscle contraction, and *in vivo* changes in blood pressure all showed similar pharmacological responses in being sensitive to the nonpeptide ligands Dup 753 and Exp 6803 and being resistant to PD 123319. It is likely, therefore, that all these responses are mediated by a similar, if not identical, subtype of the angiotensin II receptor. Furthermore, the PD 123319-sensitive site evidently does not play a role in mediating angiotensin II effects upon contractility and blood pressure.

Our data indicate that the predominant angiotensin II binding site in rabbit adrenal and rat liver is the Dup 753/Exp 6803-sensitive subtype. In uterus, the predominant subtype appears to be the PD 123319-sensitive site, although it is clear that substantial amounts of the Dup 753-sensitive site are present as well. It is also apparent that the Dup 753-sensitive site mediates contraction of uterine smooth muscle, because angiotensin II-stimulated contraction was inhibited by low concentrations of Dup 753. This also suggests a separation of functional responses for angiotensin II binding sites delineated by the nonpeptide ligands.

In summary, the recent discovery of nonpeptide ligands has led to a clear pharmacological separation of angiotensin II binding sites. It is apparent that Dup 753 and Exp 6803 are affecting a subclass of angiotensin II binding sites that are coupled to inositol phosphate metabolism and that regulate vascular contractility and blood pressure. Although no role may be currently ascribed to the binding sites that are sensitive to PD 123319, it is likely that these sites will have dissimilar signaling mechanisms and functional responses from those sensitive to Dup 753 and Exp 6803. Given the recent data on distribution of this site, it is possible that it may play a role in mediating catechol release, hypertrophy, and possibly neural

function. The identity of at least two distinct angiotensin II binding sites, both of which likely represent true receptors, and the identification of selective antagonists will be of great utility in understanding the biology of angiotensin II.

Acknowledgments

The authors wish to thank Ms. Bronia Olzewski for her surgical assistance in preparing the renal hypertensive rats and Mr. James Fergus for assistance in conducting the binding assays.

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