0026-895X/92/061081-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:1081-1088

Different Types of Receptor Interaction of Peptide and Nonpeptide Angiotensin II Antagonists Revealed by Receptor Binding and Functional Studies

WOLFGANG WIENEN, ANNEROSE B. M. MAUZ, JACQUES C. A. VAN MEEL, and MICHAEL ENTZEROTH

Department of Pharma Research, Dr. Karl Thomae GmbH, 7950 Biberach, Germany

Received August 21, 1991; Accepted March 11, 1992

SUMMARY

The pharmacological effects of angiotensin II (AII) are potently inhibited by several peptide and recently synthesized nonpeptide AII receptor antagonists. The interaction of sarcosine[1], isoleucine[8]-AII (sarile), sarcosine[1],*O*-methyltyrosine[4]-AII (sarmesin), and the nonpeptide AII antagonists 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazole-5-yl)biphenyl-4-yl)-methyl]imidazole (DuP 753, Losartan potassium) and its metabolite 2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazole-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (EXP3174) with AII binding sites was investigated in radioligand binding and functional studies. Sarile, sarmesin, DuP 753, and EXP3174 inhibited ^{125}I-AII binding to rat lung tissue, with K_i values of 3.5, 16.1, 23.7, and 10.4 nM, respectively. The Hill coefficients of all displacement curves, except for sarile (n_H, 1.45), were not significantly different from unity. In functional experiments using rabbit aorta, sarmesin and DuP 753 competitively inhibited the contractile response to AII, with pA_2 values of 6.75 and 8.01, respectively. Sarile, in contrast, revealed noncompetitive antagonism, i.e., the maximum contractile force and the slope of the concentration-contractile force curve were significantly and concentration-dependently depressed. The concentration-contractile response curve for AII was shifted to the right in a parallel fashion in the presence of EXP3174 (3 nM to 1 μM); however, the maximum contractile force was significantly decreased, by 24%. The marked noncompetitive antagonism of sarile (3 nM) was reversed in the presence of increasing concentrations of sarmesin (30 nM to 30 μM) or DuP 753 (10 nM to 1 μM), whereas in the presence of increasing concentrations of EXP3174 (3–300 nM) a 25% depression in maximum contractile force persisted. Moreover, the reduction of the maximum contractile force by EXP3174 (10 nM) was concentration-dependently restored in the presence of increasing concentrations of DuP 753 (10 nM to 1 μM), indicating interaction with the same binding site. Whereas sarile (0.3–10 nM) did not affect the ^{125}I-AII binding capacity in radioligand saturation experiments, a 54% reduction of B_{max} was observed in the presence of 100 nM EXP3174. The data provide evidence that all antagonists inhibit the functional response to AII by interacting with a common binding site at the receptor. The noncompetitive behavior of sarile seems to be due to slow dissociation from this receptor site. An additional mechanism must be postulated for EXP3174. An allosteric interaction with the receptor, as suggested by the reduction in B_{max}, may be, at least in part, responsible for the nonclassical antagonism of this compound.

The RAS plays an essential role in the homeostasis of body fluid and sodium balance and in the control of blood pressure. The introduction of angiotensin-converting enzyme inhibitors has clearly demonstrated the beneficial effect of pharmacological inhibition of the RAS in the treatment of hypertension and congestive heart failure.

Great effort has also been made during the past two decades to develop specific antagonists for AII, the effector hormone of the RAS. Until recently, all these antagonists were peptide analogs of AII, such as [Sar[1],Ala[8]]-AII (saralasin) or [Sar[1],Ile[8]]-AII (sarile) (1, 2). These peptides are potent and specific AII receptor antagonists but possess partial agonistic activity and show noncompetitive antagonism in various pharmacological models (3). More recently, a new type of AII antagonist, [Sar[1],Tyr(Me)[4]]AII (sarmesin), was found to act in a truly competitive manner (4). Further progress in our understanding of the function and classification of AII receptors has been achieved with the development of specific nonpeptide AII antagonists. Whereas the classical peptide antagonists are not able to distinguish between different AII receptors, subtypes could clearly be identified with the use of these nonpeptide structures (5, 6). With the aid of these compounds, the classification as angiotensin AT_1, sensitive to DuP 753 (Losartan potassium), and AT_2, sensitive to PD 123,177, receptors was made possible (7, 8). At present, the known pharmacological actions of AII seem to be mediated by the AT_1 receptor, which is the only subtype present in vascular smooth muscle or rat lung tissue (9, 10). A series of new nonpeptide AII antagonists,

Parts of this study have been presented at the XIth IUPHAR Congress, Amsterdam, 1990.

ABBREVIATIONS: RAS, renin-angiotensin system; AII, angiotensin II.

selective for the AT_1 subtype, has been described recently (for review, see Ref. 11). These compounds have been characterized as specific and competitive AT_1 receptor antagonists without partial agonistic activity. The lead structure of this new class of AII antagonists is represented by DuP 753, an orally active, antihypertensive, AII antagonist (12). EXP3174, an active metabolite of DuP 753, was also found to be a selective AT_1 receptor antagonist (13). Interestingly, this compound exerted noncompetitive antagonism in a nonclassical fashion, making it also a new and useful tool for the investigation of the AII receptor.

The aim of the receptor binding and functional studies was to gain insight into the different modes of receptor-ligand interaction of established peptide (sarile and sarmesin) and the recently developed, selective, nonpeptide (DuP 753 and EXP3174) AII antagonists.

Materials and Methods

Rat Lung Membrane Preparation

Rat lung membrane preparations were obtained as follows. Male Wistar (strain Chbb:THOM; 200–220 g) rats were killed by a blow on the neck. The lungs were dissected out and cleaned. The tissue was weighed and homogenized in Tris-buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.20) with an Ultra-Turrax homogenizer, at maximal setting, for 30 sec. The homogenate was centrifuged for 10 min at 1000 × *g*. The pellet was discarded, and the supernatant was recentrifuged twice for 20 min at 48,000 × *g*. The final pellet was resuspended in incubation buffer (50 mM Tris, 5 mM $MgCl_2$, 0.2% bovine serum albumin, pH 7.20).

Radioligand Binding

Protein (0.04–0.1 mg) was incubated with 1–5000 pM ^{125}I-AII for saturation experiments or 50 pM radioligand for displacement studies, with increasing concentrations of the competitors, at 37°, in a total volume of 0.2 ml. To acertain equilibrium conditions, ^{125}I-AII binding kinetics were investigated over a period of 180 min. Equilibrium was reached within 60 min of incubation. Therefore, this incubation period was chosen for all subsequent experiments. The incubation was terminated by rapid filtration through GF/B glass fiber filters, using a Skatron cell harvester. The filters were washed twice with ice-cold buffer, and particle-bound radioligand was assayed in a γ counter. Nonspecific binding was defined as radioactivity bound in the presence of 1 μM AII in the incubation medium. All samples were run in triplicate. Protein was determined according to the method of Lowry *et al.* (14). Bovine serum albumin was used as standard.

In Vitro Experiments in Rabbit Aortic Rings

Female New Zealand white rabbits (strain Chbb/NZW; 1.5 kg) were sacrificed by cervical dislocation and exsanguinated. The descending thoracic aorta was dissected free, transferred to prewarmed (37°) and oxygenated Krebs solution, and cleaned of adherent fat and connective tissue. The aorta was cut into 5-mm rings, and the rings were mounted in 30-ml organ baths, containing Krebs bicarbonate solution of the following composition (in mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 10; $CaCl_2$, 2.5. The Krebs solution was kept at 37° and pH 7.4 while being bubbled continuously with 5% CO_2 in O_2. Isometric contraction was recorded using Statham UC-2 force transducers. Initial resting tension was set to 2.0 g, and the rings were allowed to equilibrate for approximately 90 min. During this period, the rings were stimulated two times by the addition of AII to the bath, in a final concentration of 30 nM. An interval of 30 min was left between each stimulation. After the maximum contractile response was reached, the rings were rinsed three times and were allowed to relax to base-line tension. After this general procedure, the experiments were divided into three groups.

Single drug-receptor interaction. After equilibration, a control cumulative concentration-contractile response curve for AII (0.3–100 nM) was obtained. No desensitization of the tissues was observed after repeated cumulative administration of AII (data not shown). Thereafter, the tissues were washed and allowed to return to base-line tension. Each tissue was subsequently incubated for 30 min with a single concentration of the respective antagonists before a second cumulative concentration-contractile response curve for AII (1 nM to 100 μM), in the presence of antagonist, was obtained. This incubation time was considered sufficient to obtain equilibrium, because an extension up to 90 min led to identical results. Furthermore, to exclude any influence of multiple dosing with the respective antagonist on the concentration-contractile reponse curve, due to the lipophilicity and/or slow dissociation kinetics of the antagonist, each tissue was incubated with only one concentration of the antagonist. In the case of competitive antagonist-receptor interaction (sarmesin and DuP 753), the method of Arunlakshana and Schild (15) was used to calculate the pA_2 and to determine the competitive character of antagonism of the compounds.

Receptor-protection studies. An initial control concentration-contractile response curve for AII (0.3–100 nM) was obtained. Tissues were washed and tension was allowed to return to base-line values. Sarile (3 nM) or EXP3174 (10 nM), as noncompetitive antagonists, were then added to the bath, either alone or in combination with a concentration of one of the other antagonists. The tissues were equilibrated for 30 min with the compounds before a second cumulative concentration-contractile response curve was obtained.

Washout experiments with noncompetitive antagonists. After the equilibration period, the tissues were again challenged with a single AII dose, in a final concentration of 30 nM. The maximum contractile force from this challenge was taken as the control response to AII. Tissues were then incubated for 30 min with either the vehicle or one of the respective antagonists and were again challenged with AII (30 nM). Thereafter, the antagonist (or vehicle) was removed from the tissues by thorough washing. The tissues were again equilibrated for an additional 40 min, during which the medium was exchanged five times with fresh buffer. Additional challenges with AII (30 nM) were performed 40, 80, and 120 min after removal of the antagonist.

Data Evaluation and Statistics

Data are presented as mean ± standard error. Statistical analysis was performed with a one- or two-way unpaired Student's *t* test. A *p* value of <0.05 was considered to be statistically significant. Results from the functional studies are expressed as percentage of the control contractile force obtained during the first cumulative concentration-contractile response curve for AII in all sets of experiments. Binding data were analyzed with a computer-assisted nonlinear least-squares curve-fitting method, using the GraphPad (Graph Pad Software, San Diego, CA) and the RS/1 (BNN Research Systems, Cambridge, MA) software packages.

Materials

^{125}I-AII (81 TBq/mmol) was obtained from New England Nuclear (Dreieich, Germany). AII and peptide antagonists were purchased from Bachem Biochemica (Heidelberg, Germany) and Bachem Bissendorf Biochemicals (Hannover, Germany). DuP 753 and EXP3174 were synthesized by Drs. U. Ries and B. Narr in the Chemistry Department of Dr. Karl Thomae GmbH (Biberach, Germany). All other chemicals were of the best grade available.

Results

The interaction of DuP 753, EXP3174, sarile, and sarmesin with AT_1 receptors was investigated in radioligand binding experiments. In rat lung tissue, ^{125}I-AII interacted with a single population of binding sites (Fig. 1). The dissociation constant (K_d) determined in this preparation was 0.79 ± 0.15 nM. The

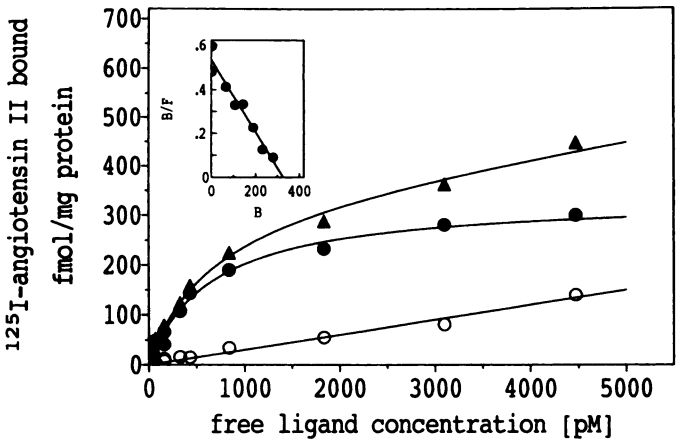


Fig. 1. Saturation binding of ^{125}I-AII to membrane preparations from rat lung. Total (▲), specific (●), and nonspecific (○) binding of ^{125}I-AII was determined for 60 min at 37°. Values are the means of a typical experiment (three determinations) done in triplicate. Nonspecific binding was determined in the presence of 1 μM unlabeled peptide. *Inset*, Scatchard plot of saturation data.

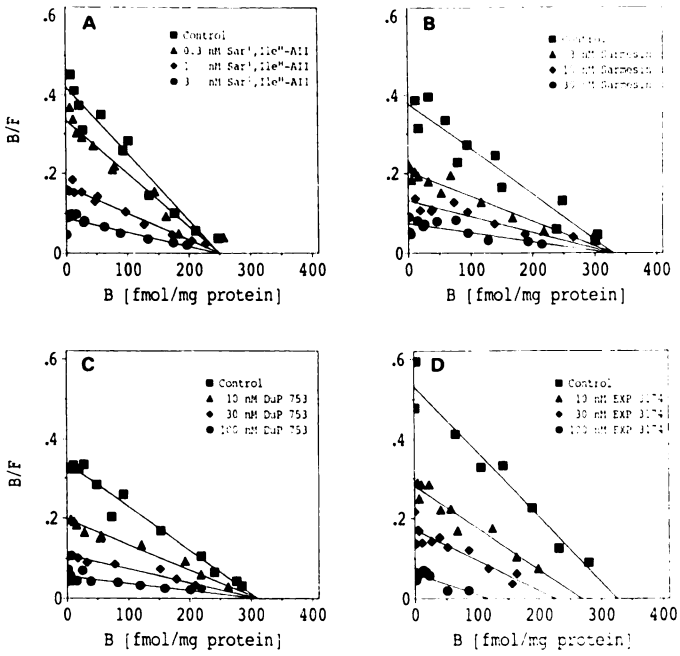
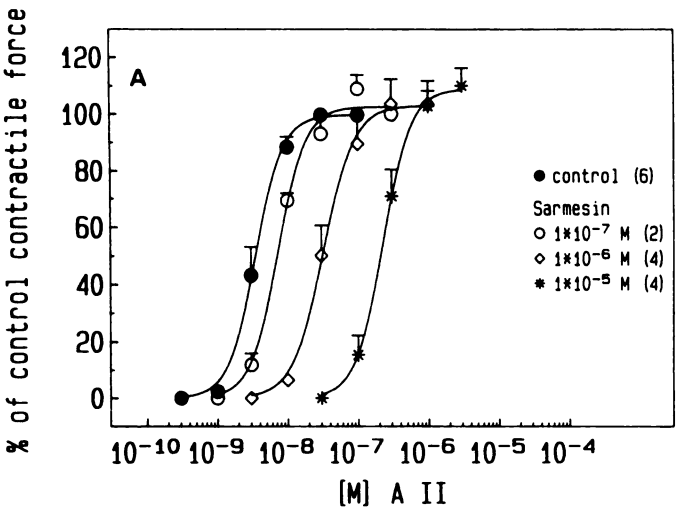


Fig. 2. Scatchard transformations of saturation binding data for ^{125}I-AII binding to membrane preparations from rat lung, in the presence of different concentrations of peptide [sarile (A) and sarmesin (B)] and nonpeptide [DuP 753 (C) and EXP3174 (D)] antagonists. The data represent the means of typical experiments run in triplicate.

corresponding number of binding sites labeled by the radioligand was 301 ± 28 fmol/mg of protein. In displacement studies using 50 pM ^{125}I-AII, the affinity of the compounds for AT_1 receptors was determined. In this preparation, all antagonists inhibited ^{125}I-AII binding, in a concentration-dependent manner. Sarile was the most potent inhibitor of radioligand binding, with an apparent dissociation constant (K_i) of 3.1 ± 1.2 nM (mean ± standard error; three experiments). Analysis of the displacement curve revealed a positive cooperative interaction with the binding sites, because the Hill coefficient was significantly different from unity (1.45 ± 0.05; $p < 0.01$). Sarmesin, another peptide antagonist, inhibited radioligand binding with an apparent dissociation constant of 16.1 ± 2.3 nM, but in contrast to sarile the Hill coefficient of the displacement curves, 0.98 ± 0.08, was not different from unity. The nonpeptide antagonists DuP 753 and EXP3174 also recognized a single class of sites. The Hill coefficients of the competition curves were 0.99 ± 0.01 and 0.88 ± 0.06, respectively, and were both not significantly different from unity. Both compounds potently inhibited radioligand binding, with K_i values of 23.7 ± 2.5 nM and 10.4 ± 0.9 nM, respectively.

We investigated the saturation binding of ^{125}I-AII in the presence of the AII antagonists sarile, sarmesin, DuP 753, and EXP3174. The results obtained are depicted in Fig. 2. Analysis of the Scatchard transformations of ^{125}I-AII saturation curves in the presence of sarile (0.3, 1, and 3 nM) revealed that the antagonist did not affect the total number of binding sites labeled by ^{125}I-AII but increased the dissociation constant of the radioligand by a factor of 1.39 ± 0.06, 3.21 ± 0.24, and 7.2 ± 1.5, respectively. Similar results were obtained for sarmesin (1, 3, 10, and 30 nM), which increased the K_d of the radioligand 1.14 ± 0.10-, 1.68 ± 0.32-, 3.09 ± 0.60-, and 4.59 ± 0.77-fold, respectively, but did not influence the B_{max}. Also, the nonpeptide antagonist DuP 753 (10, 30, and 100 nM) competitively interacted with the receptor. At the concentrations tested, DuP 753 did not affect the number of binding sites labeled by ^{125}I-AII; however, it increased the K_d of the radioligand 1.54 ± 0.18-, 2.33 ± 0.44-, and 4.72 ± 0.61-fold, respectively. In contrast to the results obtained with these antagonists, EXP3174 interacted with the receptor in a more complex manner. At concentrations of 3, 10, 30, and 100 nM EXP3174, the maximal binding capacity was concentration-dependently reduced to 97.1, 85.8, 65.9, and 46.4% of control and the K_d of the radioligand was increased by a factor of 1.26 ± 0.03, 1.81 ± 0.15, 2.81 ± 0.49, and 3.41 ± 0.49, respectively.

The potency and the nature of AII antagonism of these compounds were also investigated *in vitro*, using the contractile response of isolated rabbit aortic rings as a functional assay. Sarmesin, a peptide AII antagonist, revealed competitive antagonism in this model (Fig. 3A). The concentration-contractile response curve for AII was shifted to the right in a parallel manner, and no depression of the maximum contractile response occurred in the presence of 0.1, 1, or 10 μM levels of the compound. Analysis according to the method of Arunlakshana and Schild (15) revealed a pA_2 of 6.75, with a slope of 1.07 (r = 0.95), which was not significantly different from unity. DuP 753, the nonpeptide antagonist, also shifted the concentration-contractile response curve for AII to the right in a parallel fashion (Fig. 3B). In the presence of either 10, 30, 100, 300, or 1000 nM concentrations of the compound, no depression of the maximum contractile force could be detected. Data analysis showed a pA_2 of 8.01, with a slope of 1.04 (r = 0.99), which also was not significantly different from unity. Sarile, another peptide antagonist, exerted the well known insurmountable antagonism in this assay (Fig. 4A). The slope of the concentration-contractile response curve of AII was significantly depressed in the presence of either 1 or 3 nM peptide. In addition, the maximum response was diminished to 60% and 12% of the control contractile force, respectively. EXP3174, another DuP 753-related nonpeptide antagonist, also shifted the concentration-contractile response curve for AII to the right (Fig. 4B). In the presence of 3, 30, 100, and 1000 nM concentrations of the compound, the maximum response decreased to 89, 81, 76, and 76%, respectively, of the control contractile force. Inter-



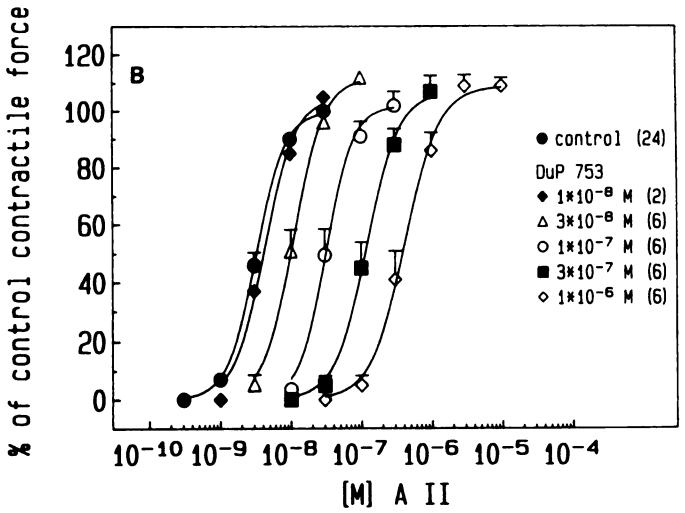
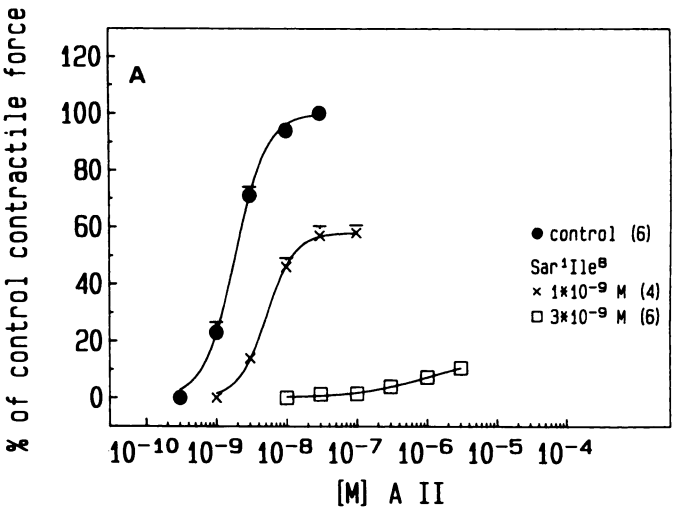


Fig. 3. Effect of the competitive AII antagonists sarmesin (A) and DuP 753 (B) on the log concentration-contractile response curve for AII in isolated rabbit aortic rings. The respective concentrations of the antagonists and the number of rings used (given in parentheses) for each concentration-contractile response curve are shown. All values are given as the mean ± standard error of the maximum contractile force obtained under control conditions before addition of antagonist.



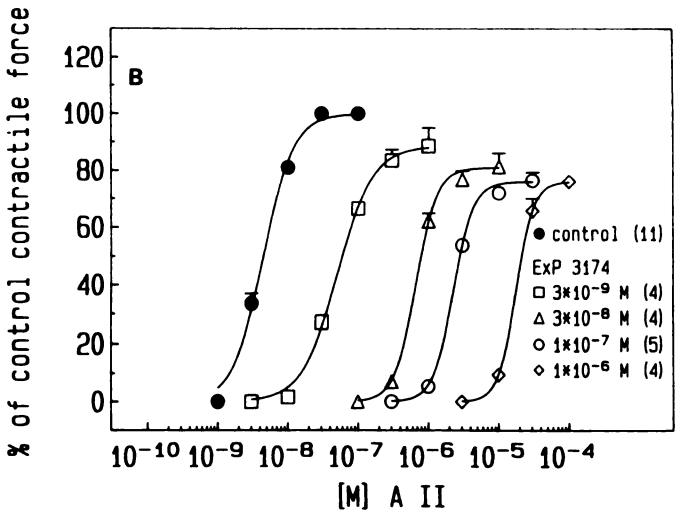


Fig. 4. Effect of the noncompetitive AII antagonists sarile (A) and EXP3174 (B) on the log concentration-contractile response curve for AII in isolated rabbit aortic rings. The respective concentrations of the antagonists and the number of rings used (given in parentheses) for each concentration-contractile response curve are shown. All values are given as the mean ± standard error of the maximum contractile force obtained under control conditions before addition of the antagonist.

estingly, the slopes of the curves were not significantly changed in the presence of increasing concentrations of EXP3174, compared with control.

Because previous studies (16, 17) suggested a slow dissociation of sarile from AII binding sites, we investigated the attenuation of the inhibitory effects of both competitive (DuP 753 and sarmesin) and noncompetitive antagonists (sarile and EXP3174), after several washout procedures. In control experiments, the response to AII, at a concentration (30 nM) that produces maximum contraction, remained stable during the entire experimental period, and no tachyphylaxis was observed (Table 1). The response to AII was significantly depressed in the presence of sarmesin (30 μM); however, a complete recovery of the contractile response was observed already after the first washout cycle. DuP 753 (0.3 μM), sarile (3 nM), and EXP3174 (10 nM) also substantially or completely inhibited the response to AII. In contrast to sarmesin, a significant reduction in the

TABLE 1

Effect of several washout cycles on the inhibition of the contractile response to AII (30 nM) by sarmesin (30 μM), DuP 753 (0.3 μM), sarile (3 nM), or EXP3174 (10 nM)

The absolute values for the pre-drug contractile responses were, for the vehicle, 4.6 ± 0.4 g; for sarmesin, 4.4 ± 0.4 g; for DuP 753, 5.9 ± 1 g; for sarile, 5.2 ± 0.5 g; and, for EXP3174, 5.1 ± 0.7 g.

	Response				
	Pre-drug	+Antagonist (or vehicle)	40-min washout	80-min washout	120-min washout
			%		
Vehicle (8)[a]	100	97 ± 2	99 ± 3	103 ± 2	103 ± 3
Sarmesin (5)	100	10 ± 2[b]	106 ± 1	108 ± 2	104 ± 1
DuP 753 (5)	100	17 ± 3[b]	33 ± 3[b]	44 ± 5[b]	60 ± 7[b]
Sarile (7)	100	0[b]	29 ± 3[b]	52 ± 4[b]	68 ± 5[b]
EXP3174 (5)	100	0[b]	6 ± 1[b]	32 ± 4[b]	51 ± 3[b]

[a] Number of experiments is given in parentheses.
[b] $p < 0.05$, versus pre-drug response.

recovery of the contractile response was observed after washout of these antagonists. Moreover, the recovery was still incomplete after the third washout cycle.

To characterize further the insurmountable antagonism of sarile at the receptor in the functional assay, we performed receptor-protection studies, by incubating the tissues with increasing concentrations of one of the other antagonists in combination with a fixed concentration of sarile (3 nM). The effect of the combined incubation with sarmesin and sarile on the contractile response in the rabbit aorta is shown in Fig. 5A. Sarile alone consistently produced strong and insurmountable antagonism. This effect was concentration-dependently reversed by the simultaneous addition of sarmesin. In the presence of 30, 300, 3,000, and 30,000 nM sarmesin, the maximum response was restored to 14, 32, 64, and 80%, respectively, of the control contractile force, and the slope was significantly shifted towards the control value. A similar effect could be observed when DuP 753 was coincubated with sarile (Fig. 5B). In the presence of 10, 30, 100, 300, and 1000 nM DuP 753, the depression of the maximum response was also concentration-dependently reversed and returned to 18, 60, 90, 97, and 107%, respectively, of the control contractile force. In additional experiments, sarile was added 30 min before DuP 753. It could be demonstrated that the maximal contractile force was restored to only 42, 60, 76, and 83% in the presence of 100, 300, 1000, and 3000 nM DuP 753, respectively (data not shown).

The effect of EXP3174, in the presence of sarile, on the contractile response is shown in Fig. 5C. The maximum response was restored to 49, 69, and 75% of the control contractile force in the presence of 3, 30, and 300 nM EXP3174, respectively. As was observed with the previous receptor-protection experiments, the slopes of the curves were again shifted towards control values by the combined incubation of EXP3174 with sarile.

In additional receptor-protection studies (Fig. 5D), we investigated the effect of increasing concentrations of DuP 753 (10–1000 nM) in the presence of 10 nM EXP3174. As observed in the previous protection experiments, the addition of the competitive antagonist resulted in a concentration-dependent increase of the maximum contractile force. In these experiments, the addition of 1 μM DuP 753 restored the maximum contractile force induced by AII to that obtained under control conditions.

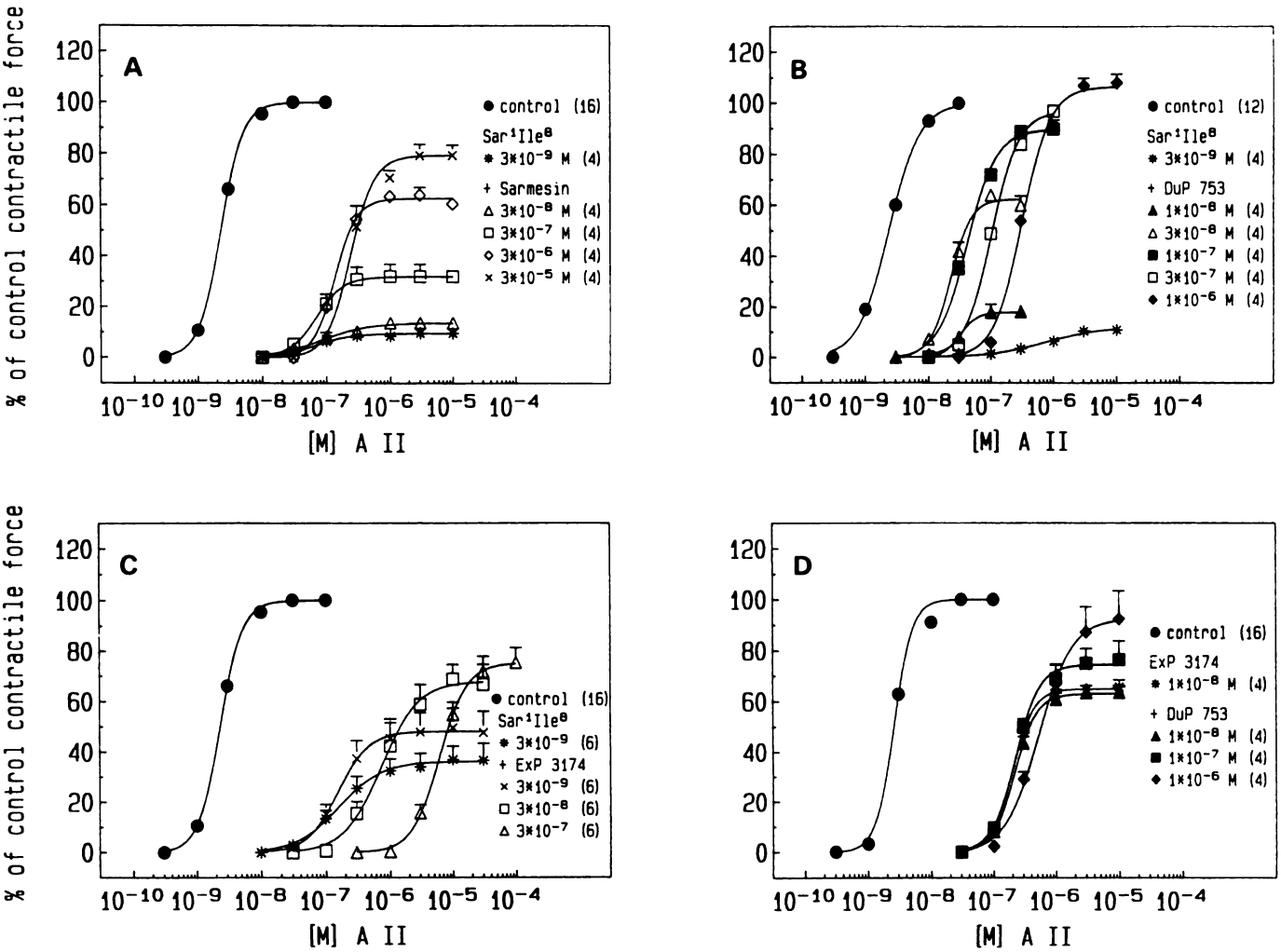


Fig. 5. Effect of coincubation with increasing concentrations of sarmesin (A), DuP 753 (B), and EXP3174 (C) in the presence of sarile (3 nM), as well as the effect of coincubation with DuP 753 in the presence of EXP3174 (10 nM) (D), on the log concentration-contractile response curve for AII in isolated rabbit aortic rings. The concentrations of the respective antagonists, alone or in combination, and the number of rings used (given in parentheses) for each concentration-response curve are shown. All values are given as the mean ± standard error of the maximum contractile response obtained under control conditions (i.e., before addition of antagonist).

Discussion

The present study describes the interaction of different peptide and nonpeptide AII antagonists with receptors in rat lung and rabbit aorta. AII receptors in both tissues are of the AT_1 subtype (10, 12, 18). The affinity of the nonpeptide antagonists for AT_1 receptors determined in radioligand binding studies is in accordance with data described in the literature (13), although EXP3174 was more potent in our hands than DuP 753. This might be due to the use of a homogeneous receptor population, as demonstrated for the tissue used in our experiments (10), in contrast to the data reported earlier. Moreover, it should be noted that the affinity of EXP3174 may vary with the assay conditions used (19). The K_i values obtained for the peptide antagonists sarile and sarmesin reflect the high potency of both compounds in functional experiments (4). All antagonists except for sarile displayed monophasic competition curves, with Hill coefficients not different from unity. Sarile, which has already been described to exhibit complex AII receptor interactions, with cooperativity in dose-response and binding curves (3), displaced the radioligand with a Hill coefficient of >1, indicating a more complex mode of receptor interaction.

In functional experiments using rabbit aortic rings, only sarmesin and DuP 753 behaved as purely competitive antagonists, as reported earlier (4, 12). Our data concerning the antagonism of sarmesin in the rabbit aorta are not in accordance with previously published results, which provide evidence for an agonistic activity of this peptide (20). The reasons for this discrepancy remain unclear but may be related to different sources of the peptide. In our experiments, sarmesin by itself, up to a concentration of 10^{-5} M, did not induce any contractile response (data not shown). In contrast to DuP 753 and sarmesin, sarile and EXP3174 exhibit insurmountable antagonism in our functional studies. The noncompetitive interaction of sarile, i.e., depression of the maximal contractile force induced by AII, with angiotensin receptors, as observed in our experiments, has already been reported previously (4). Compared with sarile, a different mode of receptor interaction has to be postulated for EXP3174. As reported by Wong *et al.* (13), we found that EXP3174 reduces the maximum response to AII. In contrast to sarile, the slopes of the concentration-response curves were not significantly depressed. This insurmountable antagonism, as expressed by sarile or EXP3174, may be evoked by several mechanisms, such as 1) multiple receptors, 2) pseudoirreversible antagonism, or 3) allosteric modulation of AII receptors (21). Furthermore, peptide AII antagonists like sarile or saralasin have intrinsic agonist activity, which might lead to receptor desensitization/internalization upon binding. This, consequently, could decrease the maximal contractile force in the isolated tissue but would not be detected in radioligand binding studies. Recently published data (22) showing that the functional response to AII in rabbit aorta after complete desensitization is restored by the addition of DuP 753 or the peptide antagonist saralasin argue against receptor internalization. Because the possibility of multiple AII receptors in the tissues used in our experiments can be excluded (10, 12, 18), we performed radioligand saturation and receptor-protection experiments to characterize further the mode of receptor interaction of the antagonists investigated. In ^{125}I-AII saturation experiments with increasing concentrations of DuP 753 or sarmesin, both antagonists decreased the dissociation constant of AII without affecting the maximum binding capacity. These results support the competitive ligand-receptor interaction already observed in functional tests. Sarile, which exhibited noncompetitive receptor interaction in functional experiments, was expected also to affect the B_{max} in binding studies. However, the antagonist competitively antagonized ^{125}I-AII binding in saturation experiments. These findings demonstrate that the insurmountable antagonism of sarile in functional tests is not likely due to an allosteric modulation of the AII receptor. Rather, a pseudoirreversible interaction of sarile with AII receptors is more likely. Such pseudoirreversible antagonism may be produced by a slow dissociation of the antagonist-receptor complex. Taylor *et al.* (23) demonstrated slow dissociation accompanied by insurmountable antagonism for ligands at the serotonin receptor. Further evidence for pseudoirreversible antagonism of sarile and a common binding site for this peptide and the competitive antagonists DuP 753 and sarmesin was provided by the receptor-protection studies. Sarmesin, as well as DuP 753, restored the maximum contractile force in response to AII in the presence of 3 nM sarile, in a dose-dependent manner. These results can be explained by the receptor binding kinetics of the compounds investigated. DuP 753 rapidly dissociates from AII binding sites, as shown in radioligand kinetic studies (24). In contrast, a slow off-rate from AII receptors has been suggested for sarile (16). Therefore, with increasing concentrations of DuP 753 or sarmesin, the fractional occupancy of the AII receptors by these compounds increases according to the law of mass action. This interpretation is supported by the finding that the shifts of the concentration-response curves at the highest concentration of DuP 753 or sarmesin used in the presence of 3 nM sarile are identical to those obtained in its absence. The receptor sites occupied by DuP 753 or sarmesin are accessible for stimulation by the agonist, whereas those occupied by sarile are not, due to a slow dissociation of the peptide from the binding site. The assumption that a slow off-rate is responsible for the noncompetitive behavior of sarile is further substantiated by our findings that about 10-fold higher concentrations of DuP 753 were needed in order to reverse the insurmountable antagonism when sarile (3 nM) was added 30 min before the addition of DuP 753, compared with simultaneous addition of both antagonists. Similar observations using receptor-protection experiments were reported for insurmountable antagonists at the serotonin receptor in the rat kidney (25).

From the washout experiments, we obtained conflicting data, which do not directly support a fast dissociation of DuP 753 from AII receptors, as reported earlier (24). The results are in agreement with data from Wong and Timmermans (26), who also described an incomplete recovery of the contractile response to AII after washout of DuP 753. It has to be taken into account that the characteristics of receptor-antagonist interaction depend on the experimental conditions. In contrast to binding studies (16, 24), factors such as lipophilicity, receptor environment, and kinetics, as well as the distribution of the antagonist in the respective tissue, may influence the washout of the antagonists. Therefore, the recovery of the contractile response after washout suggests a reversible receptor interaction for all antagonists but does not characterize the receptor off-rate.

As shown in functional studies, EXP3174 induces a parallel shift of the AII concentration-contractile response curve to the right and, at higher concentrations, reduces the contractile

force elicited by AII to maximally 24%. Comparable noncompetitive receptor interaction is also observed with several other, structurally related, AII antagonists, such as DuP 532 and EXP3892 (19, 26, 27). A common feature in the structure of these antagonists, in contrast to DuP 753, is the presence of a carboxylic function at the 5-position of the imidazole ring, which may take part in the receptor-ligand interaction. Several hypothetical models have been used to describe the type of receptor interaction exhibited by DuP 532, EXP3892, and EXP3174 (19, 26, 27). Those authors either suggested a pseudoirreversible interaction of the antagonists with AII binding sites or proposed an allosteric modulation of the receptor. As has already been demonstrated by others and by us (3, 16, 28), the noncompetitive receptor interaction of EXP3174 might also be, in part, a result of slow dissociation kinetics of the compound, as suggested by Chiu *et al.* (19). Wong and Timmermans (26) adapted the receptor-transducer coupling model of de Chaffoy de Courcelles *et al.* (29) to explain the insurmountable antagonism of EXP3892. This postulates one binding site on the outer side of the plasma membrane for the interaction with agonists and antagonists and another on the inner side of the membrane for the coupling factor. According to this model, interaction of EXP3892 or EXP3174 with the binding site on the outer side of the membrane results in a slowly reversible conformational change that diminishes the binding capacity of the coupling factor and, therefore, reduces the maximum contractile response. The data presented in this study do not favor such a model. The data from the radioligand saturation experiments suggest, rather, an allosteric interaction of EXP3174 with AT_1 receptors, which reduces the agonist binding capacity of the receptor on the outer side of the membrane. One hypothesis is that binding of EXP3174 to AT_1 receptors occurs at two sites, R and R′, as recently suggested by Moore and Scanlon (30). The agonist AII interacts with the receptor site R, resulting in a functional contractile response. Binding of the competitive antagonists DuP 753 and sarmesin, as well as the slowly dissociating antagonist sarile, to this receptor site results in the described inhibition of the functional response. The binding of EXP3174 to site R also is reversible. AII binding and pharmacological response are inhibited competitively. However, EXP3174 additionally interacts with AT_1 receptors at site R′ with high affinity, inducing a conformational change in the receptor structure that reduces the binding capacity for the agonist. DuP 753, as shown in the protection assay, also fits into the additional binding site, R′, but does not induce a conformational change. Thus, it is conceivable that, with increasing concentrations of DuP 753 in the presence of a fixed concentration of EXP3174, the number of regular binding sites, R, will increase and the functional response to AII will be restored. Alternatively, the existance of a saturable AT_1 receptor subpopulation, sensitive to EXP3174, cannot be ruled out, because the reduction in contractile response was restricted to 24% of control.

In conclusion, the results of the present study provide evidence for different modes of ligand-receptor interaction by established peptide and recently developed, selective, nonpeptide AII receptor antagonists. In particular, a nonclassical receptor binding behavior could be demonstrated for EXP3174. This phenomenon may be partly explained by the occupation of two distinct binding sites at the AT_1 receptor or two different AT_1 receptor subtypes.

Acknowledgments

The authors wish to thank Ursula Schmid and Harald Seidel for their excellent technical assistance.

References

1. Pals, D. T., F. D. Masucci, G. S. Dennings, Jr., F. Sipos, and D. C. Fessler. Role of the pressor action of angiotensin II in experimental hypertension. *Circ. Res.* **29**:673–681 (1971).
2. Türker, R. K., M. M. Hall, M. Yamamoto, C. S. Sweet, and F. M. Bumpus. A new long-lasting competitive inhibitor of angiotensin. *Science (Washington D. C.)* **177**:1203–1205 (1972).
3. Scanlon, M. N., and G. J. Moore. Classification of angiotensin receptors in rat isolated uterus, portal vein, and aorta using a slowly dissociating antagonist, [Sar^1,Ile^8]Ang II, for receptor blockade. *J. Pharmacol. Methods* **20**:207–224 (1988).
4. Scanlon, M. N., J. M. Matsoukas, K. J. Franklin, and G. J. Moore. A new approach to angiotensin antagonists: methylation of the tyrosine hydroxyl in angiotensin II. *Life Sci.* **34**:317–321 (1984).
5. 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).
6. 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).
7. 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).
8. Bumpus, F. M., K. J. Catt, A. T. Chiu, M. De Gasparo, T. Goodfriend, H. Husain, M. J. Peach, D. G. Taylor, Jr., and P. B. M. W. M. Timmermans. Nomenclature for angiotensin receptors. *Hypertension (Dallas)* **17**:720–721 (1991).
9. Murphy, T. J., K. E. Alexander, K. K. Griendling, M. S. Runge, and K. E. Bernstein. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature* (Lond.) **351**:233–236 (1991).
10. Entzeroth, M., and S. Hadamovsky. Angiotensin II receptors in the rat lung are of the AII-1 subtype. *Eur. J. Pharmacol.* **206**:237–241 (1991).
11. Johnson, A. L., D. J. Carini, A. T. Chiu, J. V. Duncia, W. A. Price, G. J. Wells, R. R. Wexler, P. C. Wong, and P. B. M. W. M. Timmermans. Nonpeptide angiotensin II receptor antagonists. *Drug News Perspect.* **3**:337–351 (1990).
12. Chiu, A. T., D. E. McCall, W. A. Price, P. C. Wong, D. J. Carini, J. V. Duncia, R. R. Wexler, E. Y. Sung, A. L. Johnson, and P. B. M. W. M. Timmermans. Nonpeptide angiotensin II antagonists. VII. Cellular and biochemical pharmacology of DuP 753, an orally active antihypertensive agent. *J. Pharmacol Exp. Ther.* **252**:711–718 (1990).
13. Wong, P. C., W. A. Price, A. T. Chiu, J. V. Duncia, D. J. Carini, R. R. Wexler, A. L. Johnson, and P. B. M. W. M. Timmermmans. Nonpeptide angiotensin II receptor antagonists. XI. Pharmacology of EXP3174: an active metabolite of DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.* **255**:211–217 (1990).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
15. Arunlakshana, A. D., and H. O. Schild. Some quantitative uses of drug agonists. *Br. J. Pharmacol.* **14**:48–58 (1959).
16. Moore, G. J., K. J. Franklin, D. M. Nystrom, and M. Goghari. Structure-desensitization relationships of angiotensin analogues in rat isolated uterus. *Can. J. Physiol. Pharmacol.* **63**:966–971 (1983).
17. De Léan, A., H. Ong, J. Gutkowska, P. W. Schiller, and N. McNicoll. Evidence for agonist-induced interaction of angiotensin receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. *Mol. Pharmacol.* **26**:498–508 (1984).
18. Criscione, L., H. Thomann, S. Whitebread, M. De Gasparo, P. Bühlmayer, P. Herold, F. Ostermayer, and B. Kamber. Binding characteristics and vascular effects of various angiotensin II antagonists. *J. Cardiovasc. Pharmacol.* **16** (Suppl. 4):S56–S59 (1990).
19. Chiu, A. T., D. J. Carini, J. V. Duncia, K. H. Leung, D. E. McCall, W. A. Price, P. C. Wong, R. D. Smith, R. R. Wexler, and P. B. M. W. M. Timmermans. DuP 532: a second generation of nonpeptide angiotensin II antagonists. *Biochem. Biophys. Res. Commun.* **177**:209–217 (1991).
20. Criscione, L., H. Thomann, S. Whitebread, M. De Gasparo, and B. Kamber. Sarmesin is a partial agonist of angiotensin-II receptors in rabbit, but not in rat, aortic rings. *Biochem. Biophys. Res. Commun.* **169**:636–642 (1990).
21. Kenakin, T. P. Drug antagonism, in *Pharmacologic Analysis of Drug Receptor Interaction* (T. P. Kenakin, ed.). Raven Press, New York, 205–244 (1987).
22. Wragg, A., M. J. Robertson, and U. L. Clark. Prevention and reversal of angiotensin II-induced tachyphylaxis in the rabbit aorta. *Br. J. Pharmacol.*, **105**:265p (1992).
23. Taylor, E. W., S. P. Duckles, and D. L. Nelson. Dissociation constants of serotonin agonists in the canine basilar artery correlate to K_i values at the 5-HT$_{1A}$ binding site. *J. Pharmacol. Exp. Ther.* **236**:118–125 (1986).
24. Chiu, A. T., D. E. McCall, P. E. Aldrich, and P. B. M. W. M. Timmermans.

[^{3}H]DuP 753, a highly potent and specific radioligand for the angiotensin II-1 receptor subtype. *Biochem. Biophys. Res. Commun.* **172:**1195–1202 (1990).
25. Bond, R. A., A. G. Ornstein, and D. E. Clarke. Unsurmountable antagonism to 5-hydroxytryptamine in rat kidneys results from pseudoirreversible inhibition rather than multiple receptors or allosteric receptor modulation. *J. Pharmacol. Exp. Ther.* **249:**401–410 (1989).
26. Wong, P. C., and P. B. M. W. Timmermans. Nonpeptide angiotensin II receptor antagonists: insurmountable angiotensin II antagonism of EXP3892 is reversed by the surmountable antagonist DuP 753. *J. Pharmacol. Exp. Ther.* **258:**49–57 (1991).
27. Timmermans, P. B. M. W. M., P. C. Wong, A. T. Chiu, and W. F. Herblin. Nonpeptide angiotensin II receptor antagonists. *Trends Pharmacol. Sci.* **12:**55–62 (1991).
28. Wienen, W., W. Diederen, A. B. M. Mauz, and J. C. A. van Meel. Reversal of the non-competitive behaviour of the angiotensin II-antagonist Sar^{1}Ile^8-AII by a nonpeptide AII-antagonist *in vitro* and *in vivo*. *Eur. J. Pharmacol.* **183:**1553 (1990).
29. de Chaffoy de Courcelles, D., J. Leysen, P. Roevens, and H. van Belle. The serotonin-S_2 receptor: a receptor transducer coupling model to explain insurmountable antagonist effects. *Drug Dev. Res.* **8:**173–178 (1986).
30. Moore, G. J., and M. N. Scanlon. Methods for analyzing and interpreting cooperativity in dose-response curves. I. Antagonist effects on angiotensin receptors in smooth muscle. *Gen. Pharmacol.* **29:**193–198 (1989).

Send reprint requests to: Wolfgang Wienen, Department of Pharmacology, Dr. Karl Thomae GmbH, P.O. Box 1755, D-7950 Biberach/Riss, Germany.