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Dual Agonistic and Antagonistic Property of Nonpeptide Angiotensin AT_1 Ligands: Susceptibility to Receptor Mutations

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SUMMARY

Two nonpeptide ligands that differ chemically by only a single methyl group but have agonistic (L-162,782) and antagonistic (L-162,389) properties *in vivo* were characterized on the cloned angiotensin AT_1 receptor. Both compounds bound with high affinity (K_i = 8 and 28 nM, respectively) to the AT_1 receptor expressed transiently in COS-7 cells as determined in radioligand competition assays. L-162,782 acted as a powerful partial agonist, stimulating phosphatidylinositol turnover with a bell-shaped dose-response curve to 64% of the maximal level reached in response to angiotensin II. Surprisingly, L-162,389 also stimulated phosphatidylinositol turnover, albeit only to a small percentage of the angiotensin response. The prototype nonpeptide AT_1 agonist L-162,313 gave a response of ~50%. The apparent EC_{50} values for all three compounds in stimulating phosphatidylinositol turnover were similar, ~30 nM, corresponding to their binding affinity. Each of the three compounds also acted as angiotensin antagonists, yet in this capacity the compounds differed markedly, with IC_{50} values ranging from 1.05×10^{-7} M for L-162,389 to 6.5×10^{-6} for L-162,782. A series of point mutations in the transmembrane segments (TMs) of the AT_1 receptor had only minor effect on the binding affinity of the nonpeptide compounds, with the exception of A104V at the top of TM III, which selectively impaired the binding of L-162,782 and L-162,389. Substitutions in the middle of TM III, VI, or VII, which did not affect the binding affinity of the compounds, impaired or eliminated the agonistic efficacy of the nonpeptides but with only minor or no effect on the angiotensin potency or efficacy. Thus, in the N295D rat AT_1 construct, L-162,782, L-162,313, and L-162,389 all antagonized the angiotensin-induced phosphatidylinositol turnover with surprisingly similar IC_{50} values (90–180 nM), and they all bound with unaltered, high affinity (22–36 nM). However, L-162,313 and L-162,782 could stimulate phosphatidylinositol turnover to only 20% of that of angiotensin. It is concluded that minor chemical modifications of either the compound or the receptor can dramatically alter the agonistic efficacy of biphenyl imidazole compounds on the AT_1 receptor without affecting their affinity, as determined in binding assays, and that a number of substitutions in the middle of the TM segments affect the efficacy of nonpeptide agonists as opposed to angiotensin.

During the past 5–7 years, nonpeptide ligands have been developed in most peptide receptor systems of the rhodopsin-like G protein-coupled type (1). Except for opioid receptor ligands, nearly all of these nonpeptide compounds have been characterized as being antagonists (2). However, this picture is currently changing, as demonstrated recently in the angiotensin system (3, 4). During the development of compounds with balanced activity on the angiotensin AT_1 and AT_2 receptors, it was discovered that a major subset of these biphenylimidazole compounds in fact acted as angiotensin agonists *in vivo* as they increased blood pressure (4). *In vitro*, these compounds, exemplified by the prototype L-162,313, bound with high affinity to the cloned AT_1 receptor and stimulated phosphatidylinositol turnover specifically through this receptor (3). In the cholecystokinin system, many receptor ligands that initially were described as antagonists have been shown to be able to also act as agonists. This has lead to the development of several series of high affinity, high potency nonpeptide agonists in this system (5, 6). These new angiotensin and cholecystokinin receptor ligands, along with the multiple high affinity nonpeptide agonists especially for the κ-opioid receptor, indicate that 7TM peptide receptors in general may eventually be targeted by nonpeptide agonists as well as by antagonists (7).

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ABBREVIATIONS: TM, transmembrane segment; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

The molecular mechanism of action of peptide versus nonpeptide ligands has been addressed by mutational analysis in several receptor systems (7, 8). In the AT_1 receptor, it was found that binding of the peptide agonist angiotensin II is dependent on a series of residues located in the exterior part of the receptor. Two acidic residues located on the same face of a putative helical extension of TM VII appeared particularly interesting (9) (Fig. 1), and it has been suggested that these two aspartic acids constitute the contact point for the important Arg2 of the ligand (10). A lysine residue located a few helical turns deep in TM V, Lys199, has been implicated as the possible interaction point for the carboxyl-terminal carboxylate group of the peptide ligand (10–13). In contrast, mutations that affect the binding of nonpeptide ligands are located deeper between the TMs, especially in TMs III, VI, and VII (14–16). In this area of the receptor, an impressive gain-of-function with respect to binding affinity for nonpeptide antagonists was obtained by Sandberg *et al*. (17) in the previously unresponsive *Xenopus laevis* AT_1 receptor through systematic substitution and combination of residues from the human receptor. In an initial search for possible interaction points for the newly discovered nonpeptide agonist, L-162,313, we found, surprisingly, that its binding was affected by neither mutations that reduced binding of the structurally homologous biphenylimidazole nonpeptide antagonists nor mutations that affected the binding of the functionally related peptide agonist (3).

In the current study, we used cells expressing the cloned AT_1 receptor to characterize binding and functional properties of two new nonpeptide compounds that differ structurally only by a single methyl group. Despite their structural similarity, these compounds behave very differently in *in*

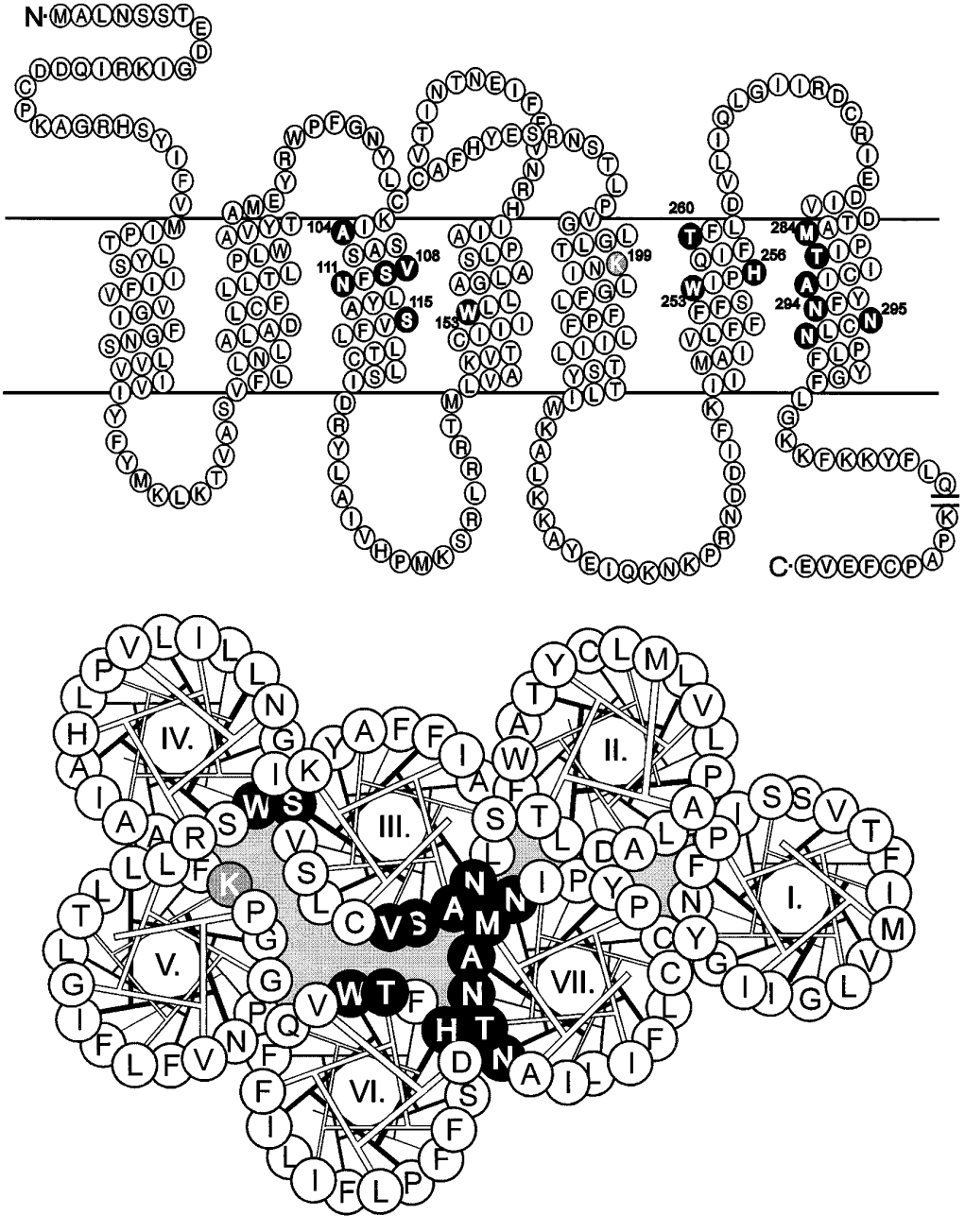

Fig. 1. *Top*, serpentine model of the human AT_1 receptor. *Bottom*, helical wheel model of the human AT_1 receptor. The helical wheel model is built over the rhodopsin structure (38) with TM III as the central helix and in an anticlockwise orientation, as viewed from an outside-in view of the receptor (8, 39). *White letters*, residues that were studied by mutagenesis in the current study; *white K against gray*, Lys199 in TM V (discussed in text).

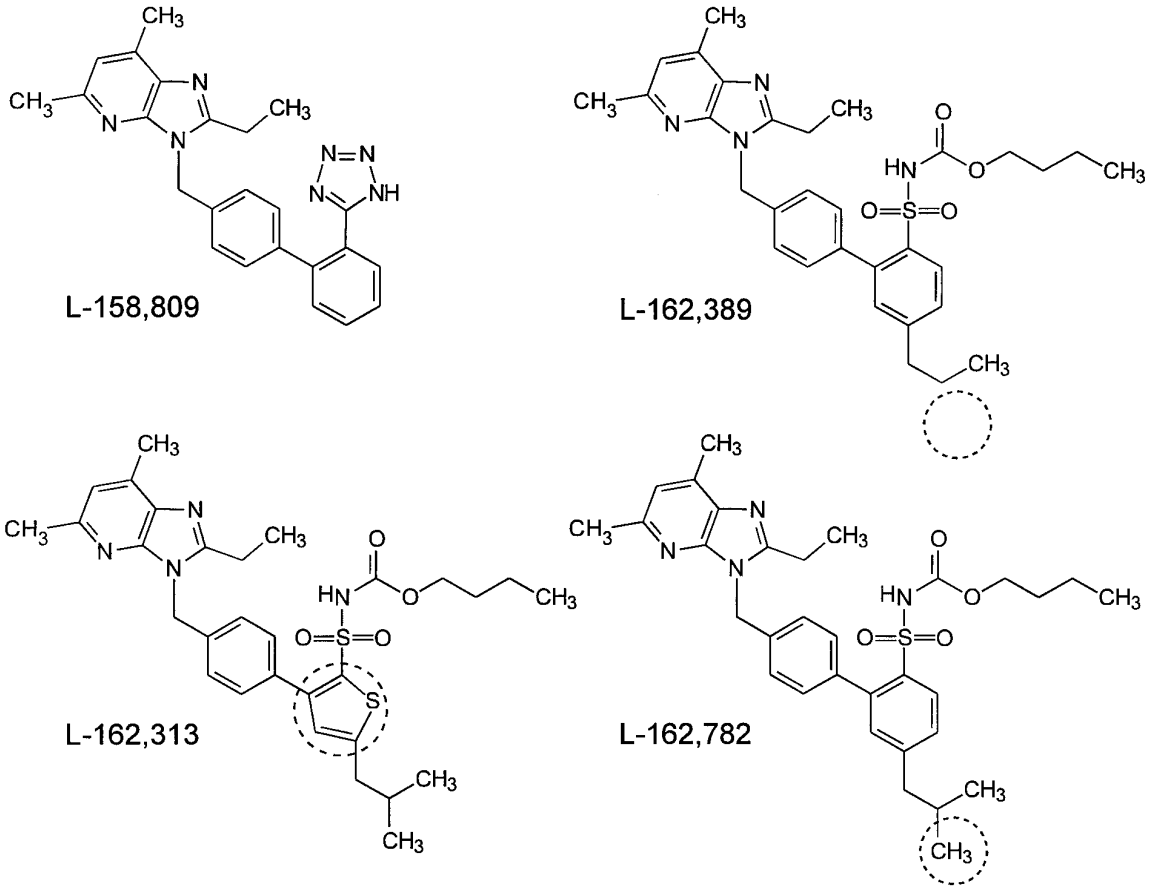

Fig. 2. Structures of nonpeptide AT_1 ligands. L-158,809, the prototype biphenylimidazole nonpeptide antagonist. L-162,313, the prototype nonpeptide agonist. L-162,389 and L-162,782, two new biphenylimidazole compounds that *in vivo* act as antagonist and agonist, respectively. Note that these compounds differ structurally only by a single methyl group (*dotted circle*) and that the only difference between L-162,313 and L-162,782 is a thiophene-for-phenyl substitution.

vivo assays because one is an angiotensin receptor agonist and the other is an antagonist.[1] These compounds and the prototype nonpeptide agonist L-162,313 were tested regarding binding properties in a library of AT_1 receptors with different point mutations in the TMs and regarding signal transduction in three mutants with point substitutions located in TMs III, VI, and VII, respectively (Fig. 1). These mutations have previously been shown to affect the binding of a variety of biphenylimidazole compounds that are pure antagonists (14, 15).

Materials and Methods

Nonpeptide and peptide ligands. The nonpeptide compounds L-162,313, L-162,389, L-162,782, and L-158,809 were synthesized as described previously (4, 18, 19) (Fig. 2). Angiotensin II and [Sar^1,Leu^8]angiotensin II were purchased from Peninsula Laboratories (Belmont, CA).

Receptor mutagenesis. The human AT_1 receptor cDNA (20) was generously provided by Dr. D. J. Bergsma (SmithKline Beecham, King of Prussia, PA), and the rat AT_1 receptor cDNA (21) was generously provided by Dr. T. J. Murphy (Emory University, Atlanta, GA). A cassette gene was initially generated of the rat receptor cDNA (9). Mutations were introduced by the PCR overlap extension technique as described previously (9, 22). The PCR fragments were digested with appropriate restriction enzymes and subsequently inserted into the likewise digested expression vector pTEJ8 (23). *Pfu* polymerase (Stratagene, La Jolla, CA) was used for the PCR reactions under reaction conditions recommended by the manufacturer. Temperature cycling consisted of 30–35 cycles at 94° for 1 min, 45–50° for 1 min, and 72° for 1 min. All receptor constructs were initially identified by the presence of a diagnostic restriction site and subsequently verified by dideoxynucleotide sequencing (Sequenase Kit, United States Biochemical, Cleveland, OH).

Cell culture and transfections. Expression plasmids containing wild-type and mutated AT_1 receptors were transiently transfected into COS-7 cells by a calcium phosphate precipitation method as described previously (24).

Phosphatidylinositol turnover. COS-7 cells ($0.15–0.4 \times 10^6$) expressing the rat AT_1 receptor or the V108A, H256A, or N295D mutant versions of this were cultivated for 24 hr in inositol-free medium (1885 Dulbecco with NaH_2CO_3, supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.1 mg/ml gentamicin) in 6- or 12-well plates, with each well containing 5 μCi of *myo*-[^{3}H]inositol (Amersham, Arlington Heights, IL), as described previously (25). The cells were washed twice with buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4) and subsequently incubated for 30 min at 37° with the same buffer including 10 mM LiCl. Dose-response experiments were performed with the nonpeptide ligands either alone or in the presence of a submaximal dose of angiotensin II with the nonpeptide compound added 30 min before the angiotensin. The reaction was terminated after 1 hr of incubation by the addition of 0.5 ml of 10% perchloric

[1] R. A. Rivero and W. J. Greenlee, unpublished observations.

acid, and the precipitated cellular proteins were removed by centrifugation. The supernatants were neutralized with 200 μl of buffer of 4.5 M KOH and 67.5 mM HEPES and incubated for 30 min with 2 ml of water and 0.5 ml of an anion exchanger, BioRad (Hercules, CA) AG 1-X8 Resin (26). The resin was washed three times with 5 mM *myo*-inositol, and the generated [^{3}H]inositol phosphates were eluted by the addition of 1 ml of 1.0 M ammonium formate in 0.1 M formic acid. The data were analyzed by computerized nonlinear regression analysis using InPlot 4.0 (GraphPAD Software, San Diego, CA).

Binding experiments. Monoiodinated ^{125}I-[Sar^1,Leu^8]angiotensin II was prepared by the Iodo-Gen method and purified by reverse-phase high performance liquid chromatography, using a gradient of 17–29% acetonitrile as described previously (14). One day after transfection and 24 hr before the binding experiments, the transfected cells were transferred to 6-, 12-, or 24-well culture plates, with 0.15–9 $\times$ 10^5 cells/well, with a goal of total binding of 5–10% of the radiolabeled peptide. The cells were washed twice with buffer (25 mM Tris, 5 mM $MgCl_2$, 140 mM NaCl, pH 7.4) before and after the binding. The binding was carried out for 24 hr at 4° with 50 pM ^{125}I-[Sar^1,Leu^8]angiotensin II and variable amounts of unlabeled nonpeptide or peptide ligands in 0.5–1 ml of a 25 mM Tris buffer containing 5 mM $MgCl_2$, pH 7.4. The binding data were analyzed by computerized nonlinear regression analysis using InPlot 4.0.

Results

Phosphatidylinositol turnover in the wild-type AT_1 receptor. The effect of the structurally closely related nonpeptide compounds L-162,782 and L-162,389 (see Fig. 2) was studied in parallel with the prototype agonist L-162,313 in COS-7 cells expressing the rat AT_1 receptor. L-162,389, which *in vivo* is an angiotensin antagonist, behaved as an insurmountable antagonist of the angiotensin-induced phosphatidylinositol turnover as it both shifted the dose-response curve for the peptide to the right and suppressed the maximally achievable response (Fig. 3). We previously found that L-162,313 also is an insurmountable angiotensin antagonist on the AT_1 receptor in stably transfected CHO cells (3).

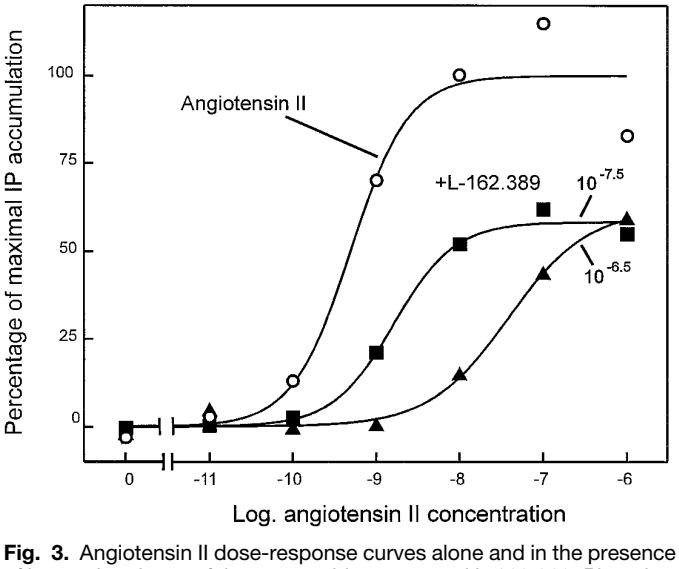

Fig. 3. Angiotensin II dose-response curves alone and in the presence of increasing doses of the nonpeptide compound L-162,389. Phosphatidylinositol turnover was measured in COS-7 cells transiently transfected with the wild-type rat AT_1 receptor. A single, representative experiment performed in duplicates is shown in which angiotensin was used (○) alone or in the presence of L-162,389 (●, $10^{-7.5}$ M; ▲, $10^{-6.5}$ M).

When applied alone to cells transfected with the AT_1 receptor, all three nonpeptide compounds were able to increase phosphatidylinositol turnover with bell-shaped dose-response curves, although the maximal responses were very different (Fig. 4). Compound L-162,782, which was known from *in vivo* studies to be an AT_1 nonpeptide agonist, functioned as the most efficient agonist as it stimulated phosphatidylinositol turnover to 64 ± 3% of the maximal level reached during stimulation with angiotensin II (Fig. 4, *top*, ■). The maximal stimulation in response to the L-162,313 compound was 47 ± 4% of the angiotensin II response, which

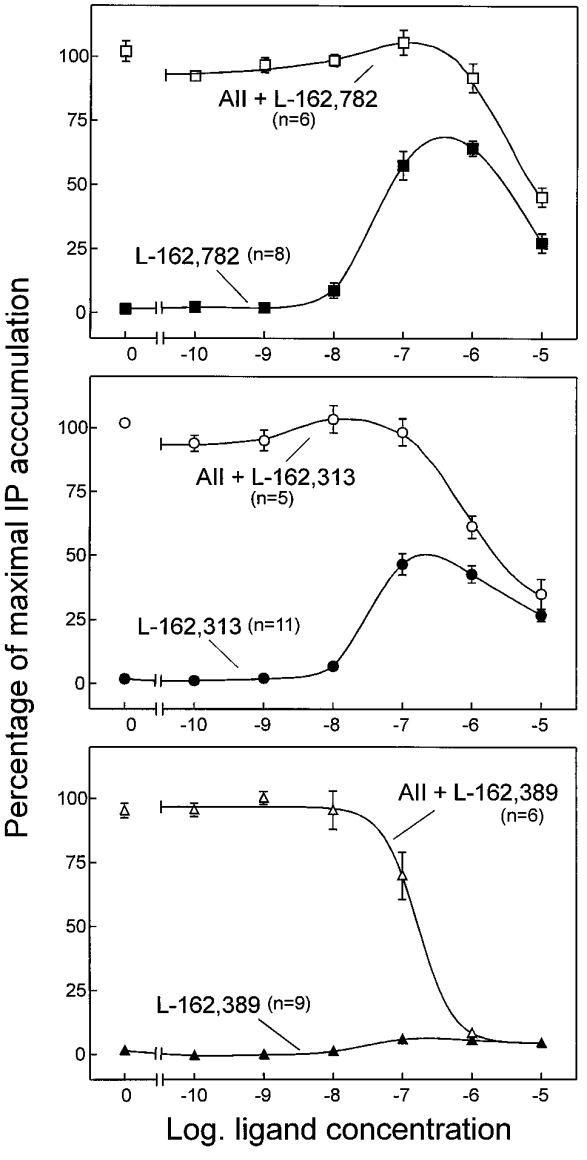

Fig. 4. Phosphatidylinositol turnover in response to nonpeptide compounds administered alone or with angiotensin II (*All*) in transiently transfected COS-7 cells expressing the rat AT_1 receptor. The phosphatidylinositol turnover is expressed as percentage of the maximal response obtained during stimulation with angiotensin II (2.9 ± 0.3 fmol of inositol/min/10^5 cells; ~3640 cpm/10^5 cells; 16 experiments; B_{max} = 72 fmol/10^5 cells). *Closed symbols*, dose-response curves for the three nonpeptide compounds when administered alone (mean ± standard error); *open symbols*, dose-response curves for the three nonpeptide compounds when given with angiotensin II ($10^{-8.5}$ M); *parentheses*, number of individual experiments performed in duplicate.

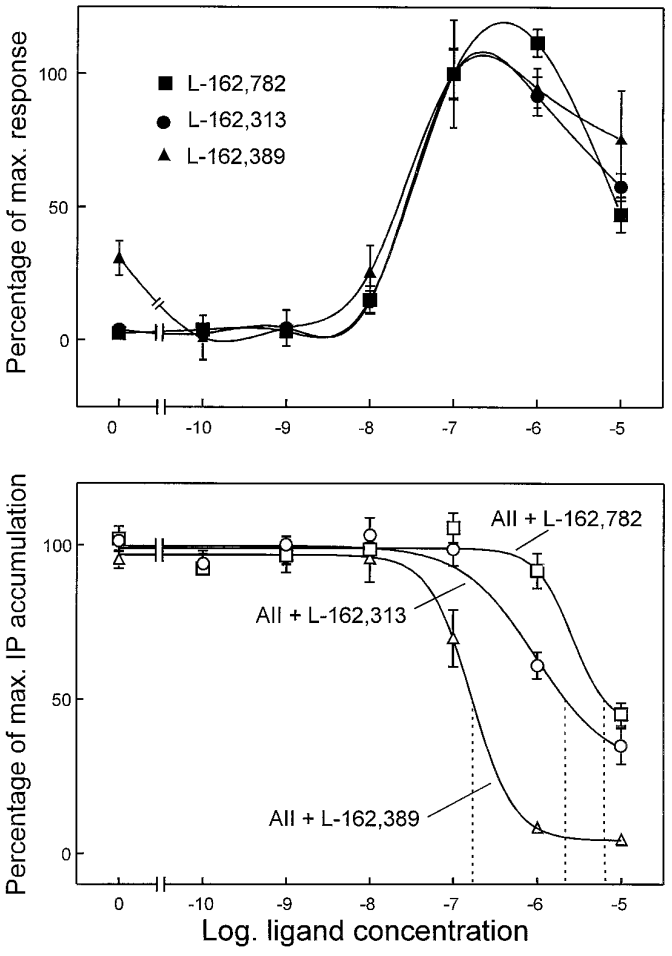

Fig. 5. Comparison of normalized stimulatory and inhibitory dose-response curves for the three nonpeptide "dualists" in the wild-type AT_1 receptor. *Top*, "agonistic" dose-response curve for the three nonpeptide compounds in stimulating phosphatidylinositol (*IP*) turnover when given alone (see legend to Fig. 3). The responses are expressed on a relative scale as percentage of the responses obtained during stimulation with 10^{-7} M concentration of the compounds (L-162,782, 2330 cpm/10^5 cells; L-162,313, 1710 cpm/10^5 cells; L-162,389, 211 cpm/10^5 cells). *Bottom*, "antagonistic" dose-response curve for the three nonpeptide compounds given with angiotensin II ($10^{-8.5}$ M).

is in agreement with previously published results (3). Based on *in vivo* studies of the direct effect on blood pressure in rats,[2] L-162,389 was not anticipated to would exhibit agonistic properties. However, in the COS-7 cells expressing the AT_1 receptor, this compound did in fact stimulate phosphatidylinositol turnover, albeit with a maximal response of only 5.8 ± 1.3% of the angiotensin II response. As shown in Fig. 5, *top*, when normalized to their individual maximal responses, the bell-shaped dose-response curves were in fact surprisingly similar for all three compounds, with an EC_{50} of ~30 nM. The actual maximal response was reached at a concentration between 10^{-7} and 10^{-6} M and was therefore in fact slightly larger than indicated above (Fig. 4).

A possible antagonistic effect of the nonpeptide compounds was studied in the transfected COS-7 cells during submaximal stimulation with angiotensin II ($10^{-8.5}$ M). As shown in Fig. 4 (*open symbols*), all three nonpeptide compounds inhibited the angiotensin-induced phosphatidylinositol turnover in a dose-dependent manner. However, in contrast to their relative similarity in regard to being agonists (Fig. 5, *top*), the nonpeptide compounds showed rather different apparent potencies as antagonists in the wild-type AT_1 receptor (Fig. 5, *bottom*). In this assay, the compound L-162,389, which displayed the least agonistic effect when applied alone, was the most potent one with an IC_{50} value of 1.05×10^{-7} M. The two more efficacious agonists, L-162,313 and L-162,782, also antagonized the angiotensin II effect but had higher IC_{50} values (2.2×10^{-6} and 6.5×10^{-6} M, respectively) (Figs. 4 and 5).

In conclusion, the three nonpeptide AT_1 ligands L-162,782, L-162,389, and L-162,313, which chemically are closely related, display both agonistic and antagonistic properties in the transfected cells expressing the wild-type AT_1 receptor, although their efficacy as agonists and their apparent potency as antagonists vary considerably.

Radioligand competition binding experiments. The peptide antagonist ^{125}I-[Sar^1,Leu^8]angiotensin II was used as radioligand for the wild-type human and rat AT_1 receptors as well as for a library of mutant human and rat receptors with point substitutions in the TMs. The three nonpeptide compounds with agonistic properties were tested along with the classic biphenylimidazole antagonist L-158,809, the peptide agonist angiotensin II, and the unlabeled peptide antagonist [Sar^1,Leu^8]angiotensin II. Some of the point mutations (e.g., those located deep in TM VII) were known from previous studies to affect the binding of nonpeptide ligands such as L-158,809 (14). In the current study, these mutations were supplemented with point substitutions, especially along the presumed inward face of the exterior part of TM VII, as well as with substitutions in the outer part of the opposing face of TM III (Fig. 1).

In the wild-type human and rat AT_1 receptors, the nonpeptide compounds L-162,389, L-162,782, and L-162,313 showed similar affinities in radioligand competition binding assays with IC_{50} values of 4–28 nM; L-162,389 had the highest affinity (Table 1). For comparison, the classic biphenylimidazole antagonist L-158,809 had an affinity of ~0.1 nM in this system (Table 1).

As previously reported (3, 14), a number of substitutions in TMs III (N111A) and VII (N294A, N295D, N298A) impaired the binding of the pure antagonist L-158,809 by 6–18-fold (Table 1). Minor differences may be noted compared with the previous analysis because the binding experiments with these mutants were performed in the absence of bovine serum albumin to optimize the assay for the nonpeptide agonists. The 8-fold reduction in the affinity of L-158,809 in response to the A104V substitution in TM III has not previously been reported (Table 1). Importantly, the binding of L-158,809 was unaffected by the new substitutions in the outer portion of TM VII; in contrast, the binding of angiotensin II was impaired 7–16-fold by individual substitution of the three residues at the top of TM VII (M284A, T287A, and A291G) (Table 1). As previously reported, angiotensin II binding is not affected by the mutations further down in TMs III and VII that impair the binding of nonpeptide antagonists (3, 14). The peptide antagonist [Sar^1,Leu^8]angiotensin II was not affected by substitutions in the middle or at the top of TM VII (Table 1).

The binding of L-162,389, the nonpeptide compound that was most potent in inhibiting the angiotensin-induced phosphatidylinositol turnover and that showed the least agonistic

[2] R. A. Rivero and W. J. Greenlee, unpublished observations.

TABLE 1

Comparison of binding affinities for antagonists in wild-type and mutated AT_1 receptors

	[Sar^1,Leu^8]Angiotensin II			Angiotensin II			L-158,809			L-162,313			L-162,389			L-162,782		
	IC_{50}	n	F_{mut}	IC_{50}	n	F_{mut}	IC_{50}	n	F_{mut}	IC_{50}	n	F_{mut}	IC_{50}	n	F_{mut}	IC_{50}	n	F_{mut}
	nM			M			nM			nM			nM			nM		
Rat AT_1 wild-type	0.34 ± 0.05	7		0.84 ± 0.10	8		0.15 ± 0.02	6		16.7 ± 2.7	12		7.72 ± 2.29	3		28.5 ± 2.2	3	
Human AT_1 wild-type	0.25 ± 0.04	20		0.52 ± 0.11	19		0.06 ± 0.01	19		16.1 ± 1.7	20		3.97 ± 0.69	16		24.6 ± 2.3	17	
TM III																		
A104V human AT_1	0.88 ± 0.27	6	3.5	1.19 ± 0.48	6	2.3	0.49 ± 0.14	5	7.8	46.5 ± 12.3	7	2.9	104 ± 33	6	26	480 ± 163	6	20
V108A rat AT_1	0.35 ± 0.02	5	1.0	1.10 ± 0.16	4	1.3	0.08 ± 0.01	3	0.5	17.7 ± 2.2	5	1.1	3.09 ± 0.47	3	0.4	27.9 ± 1.4	3	1.0
S109A human AT_1	0.79 ± 0.27	3	3.1	1.72 ± 0.62	3	3.3	0.08 ± 0.01	3	1.2	12.5 ± 1.8	3	0.8	6.20 ± 2.17	3	1.6	16.0 ± 1.5	3	0.7
N111A human AT_1	0.28 ± 0.10	4	1.1	0.25 ± 0.06	4	0.5	1.10 ± 0.22	3	18	15.7 ± 1.7	4	1.0	15.5 ± 1.5	5	3.9	31.8 ± 4.5	5	1.3
S115A human AT_1	0.48 ± 0.11	3	1.9	0.18 ± 0.10	3	0.4	0.10 ± 0.03	3	1.6	22.1 ± 5.5	4	1.4	21.4 ± 3.7	3	5.4	34.5 ± 9.2	4	1.4
TM IV																		
W153A human AT_1	0.24 ± 0.09	3	1.0	0.17 ± 0.04	3	0.3	0.04 ± 0.005	3	0.6	11.5 ± 3.9	3	0.7	6.74 ± 1.66	3	1.7	20.2 ± 3.4	3	0.8
TM VI																		
W253A human AT_1	0.36 ± 0.14	3	1.4	1.97 ± 0.71	3	3.8	0.03 ± 0.01	3	0.6	13.9 ± 1.6	3	0.9	2.61 ± 0.79	3	0.7	26.6 ± 1.0	3	1.1
H256A rat AT_1	0.77 ± 0.15	3	2.3	3.90 ± 0.75	3	4.7	0.12 ± 0.02	3	0.8	16.4 ± 1.6	3	1.0	4.67 ± 0.10	3	0.6	22.0 ± 2.5	3	0.8
T260A human AT_1	0.46 ± 0.26	4	1.8	0.34 ± 0.13	3	0.7	0.09 ± 0.01	3	1.5	15.7 ± 3.6	3	1.0	6.39 ± 1.17	3	1.6	32.4 ± 9.8	4	1.3
TM VII																		
M284A human AT_1	0.34 ± 0.16	3	1.4	3.97 ± 1.34	3	7.6	0.03 ± 0.003	3	0.5	13.6 ± 3.1	3	0.9	0.80 ± 0.07	3	0.02	15.6 ± 2.7	3	0.6
T287A human AT_1	0.89 ± 0.26	6	3.5	8.13 ± 4.28	4	16	0.10 ± 0.03	4	1.6	22.5 ± 1.2	3	1.4	31.6 ± 7.3	6	8.0	25.6 ± 4.3	6	1.0
A291G human AT_1	0.17 ± 0.05	3	0.7	3.82 ± 1.63	3	7.3	0.16 ± 0.03	3	2.5	19.1 ± 6.1	4	1.2	7.03 ± 2.24	3	1.8	33.0 ± 1.0	3	1.3
A291S human AT_1	0.56 ± 0.29	4	2.2	2.56 ± 1.45	4	4.9	0.18 ± 0.10	3	2.9	40.1 ± 6.1	4	2.5	24.6 ± 5.1	3	6.2	51.3 ± 18.8	4	2.1
N294A human AT_1	0.25 ± 0.07	4	1.0	0.52 ± 0.05	4	1.0	0.37 ± 0.16	4	5.9	15.2 ± 2.2	5	0.9	34.1 ± 6.1	3	8.6	28.1 ± 1.6	3	1.1
N295D rat AT_1	0.36 ± 0.02	5	1.1	0.44 ± 0.11	5	0.5	2.29 ± 0.69	3	15	22.5 ± 1.8	6	1.3	20.9 ± 0.3	3	2.7	36.9 ± 3.7	3	1.3
N298A rat AT_1	0.14 ± 0.02	3	0.6	0.49 ± 0.08	3	0.9	0.61 ± 0.26	3	9.8	14.9 ± 2.1	3	0.9	23.5 ± 2.5	3	5.9	26.9 ± 1.8	3	1.1

F_{mut} is calculated as IC_{50} (mutant receptor)/IC_{50} (wild-type AT_1 receptor). This corresponds to fold decrease in affinity.

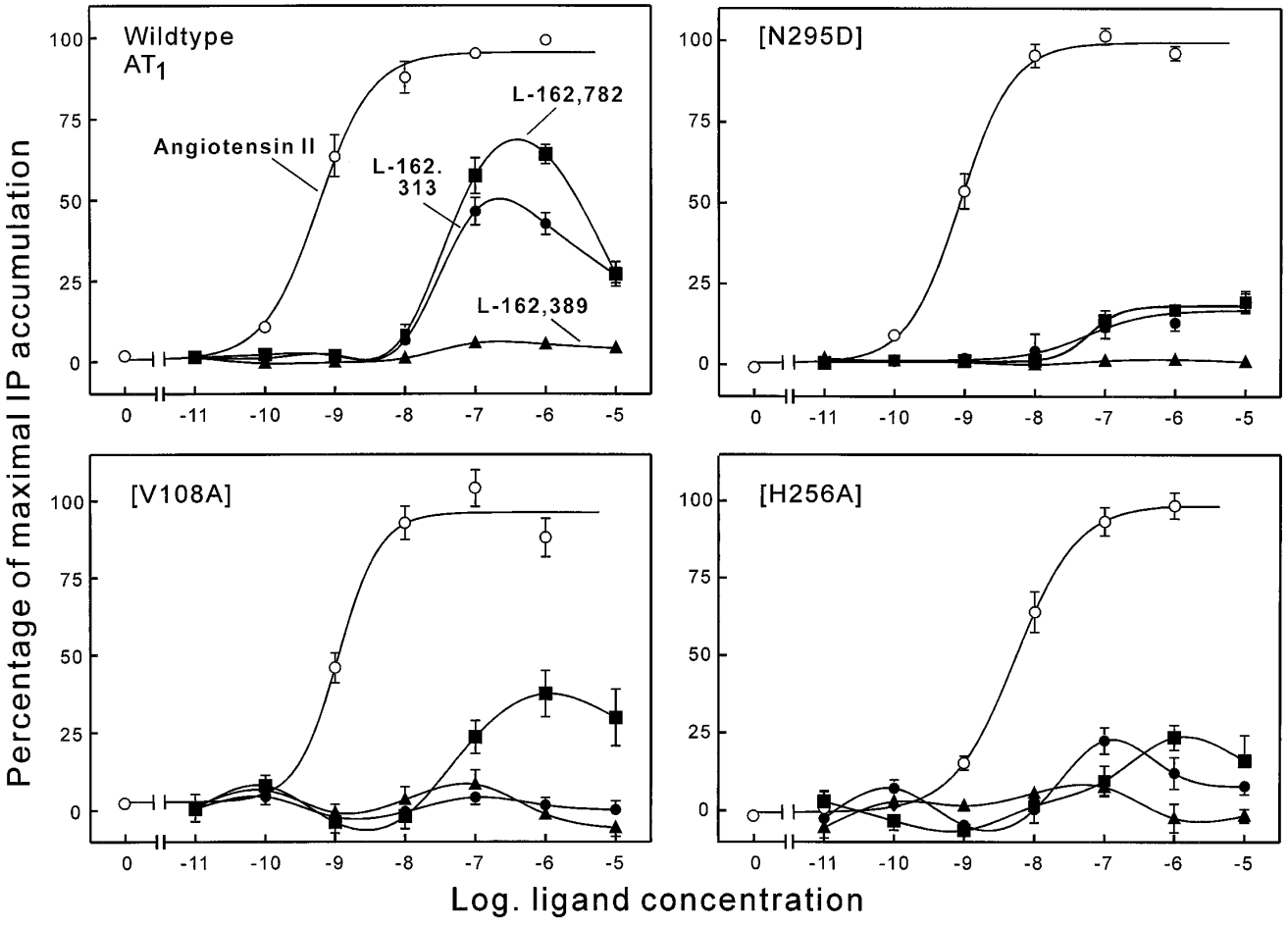

Fig. 6. Phosphatidylinositol (*IP*) turnover in response to angiotensin and nonpeptide compounds in cells expressing the rat AT_1 receptor or constructs with mutations in the TMs. Dose-response curves for angiotensin II, L-162,782, L-162,313, or L-162,389 in COS-7 cells transiently transfected with the wild-type rat AT_1 receptor [E_{max} = 3.0 ± 0.3 fmol of inositol/min/10^5 cells (3690 cpm/10^5 cells); B_{max} = 72 ± 18 fmol/10^5 cells] or the V108A construct [E_{max} = 2.1 ± 0.6 fmol of inositol/min/10^5 cells (2580 cpm/10^5 cells); B_{max} = 40 ± 16 fmol/10^5 cells], the H256A construct [E_{max} = 1.3 ± 0.8 fmol of inositol/min/10^5 cells (1600 cpm/10^5 cells); B_{max} = 154 ± 41 fmol/10^5 cells], or the N295D construct [E_{max} = 1.7 ± 0.2 fmol of inositol/min/10^5 cells (2100 cpm/10^5 cells); B_{max} = 40 ± 9 fmol/10^5 cells).

properties, was slightly (i.e., <10-fold) affected by several of the substitutions in the TMs (Table 1). The spectrum of mutations that affected L-162,389 binding overlapped both mutations that reduced angiotensin binding (T287A) and mutations that impaired the binding of the pure antagonist L158,809 (N294A) at the top and middle of TM VII, respectively (Table 1). The binding of L-162,389 was most seriously affected (26-fold) by the A104V substitution at the top of TM III, which also impaired L-158,809 binding by 8-fold. The binding of L-162,782, the nonpeptide compound that was the most efficient agonist on the wild-type receptor, was also affected seriously by the A104V mutation (20-fold) but not by any of the other mutations (0.6–2.1-fold reduction in affinity) (Table 1). The binding of the prototype AT_1 nonpeptide agonist L-162,313 was not impaired (0.7–2.9-fold reduction) by any of the substitutions in the TMs (Table 1).

Thus, except for the A104V substitution, none of the tested mutations in the TM of the AT_1 receptor had any major effect on the affinity of the nonpeptide partial agonist compounds as determined in competition binding assays, albeit slight effects were observed for the most antagonistic one, L-162,389.

Phosphatidylinositol turnover in the V108A, H256A, and N295D rat AT_1 receptors. A poor signal transduction efficiency of the human version of the AT_1 receptor in both transiently transfected COS-7 cells and stably transfected clones of CHO cell prevented a detailed investigation of the functional effect of most of the mutations of the current study because these had been performed in the human receptor. However, in the rat AT_1 receptor and in the three mutant form that we presented, angiotensin II was able to induce a considerable and reproducible increase in phosphatidylinositol turnover (Fig. 6).

In accordance with the lack of effect of the mutants on angiotensin II affinity as determined in binding assays, the EC_{50} for the peptide was similar in the wild-type AT_1 receptor (0.6 nM) and the mutants with either the V108A substitution in TM III (1.0 nM) or the N295D substitution in TM VII (0.9 nM). In the mutant with the H256A substitution in TM VI, the dose-response curve for angiotensin was shifted 10-fold to the right (EC_{50} = 6 nM; Fig. 6). The E_{max} value in response to angiotensin II stimulation was similar in the wild-type and the various mutant receptors, although a

slight but parallel reduction in both E_{max} and B_{max} values was observed in both the H256 and N295D constructs (see legend to Fig. 6).

In contrast to angiotensin, the agonistic efficacy of both L-162,782 and L-162,313 was impaired in all three mutant rat receptors with substitutions in the TMs. The effect was most pronounced for L-162,313, which was unable to stimulate phosphatidylinositol turnover in the V108A mutant and showed only a much reduced response in the N295D construct (Fig. 6). As in the wild-type receptor, no clear stimulation was observed in response to L-162,389 in any of the mutant receptors (Fig. 6).

The effects of the nonpeptide compounds were studied in greater detail in the N295D rat AT_1 construct (Fig. 7). In this mutant, the increase in phosphatidylinositol turnover in response to L-162,782 and L-162,313 amounted to only 19.4 ± 2.6% and 19.4 ± 3.4% of the angiotensin response, respectively (Figs. 6 and 7). Interestingly, no clear bell-shaped dose-response curves were observed in the N295D rat AT_1 construct for the nonpeptide compounds that here functioned as merely relatively poor, classic partial agonists (Fig. 7). In regard to antagonistic property, all three nonpeptide compounds surprisingly acted as equally potent inhibitors of the angiotensin-induced increase in phosphatidylinositol turnover in the N295D construct, with IC_{50} values of 90–180 nM (Fig. 7). This is in contrast to the almost 100-fold difference in potency as antagonists of these compounds on the wild-type receptor (Figs. 4 and 5). Thus, in the N295D construct, there was a relatively close relationship between the affinity of the compounds as determined in competition binding assays (IC_{50} = 21–37 nM; Table 1) and the IC_{50} values obtained for these compounds as antagonists for a submaximal stimulatory dose of angiotensin (Fig. 7). In the N295D construct, the L-162,389 behaved as an insurmountable antagonist as it did in the wild-type AT_1 receptor (data not shown), which unfortunately complicates a more thorough analysis of antagonist potency.

Thus, several point substitutions in the middle of TMs III, VI, and VII of the AT_1 receptor either impaired or eliminated the agonistic efficacy of the nonpeptide compounds without affecting the affinity of the compounds as determined in competition binding assays and importantly without affecting either binding or action of angiotensin.

Discussion

In the current study, we found that a series of biphenylimidazole compounds have dual agonistic and antagonistic properties on the angiotensin AT_1 receptor and that the absence or presence of just a single methyl group may dramatically change their activity from being predominantly inhibitory to being predominantly stimulatory, with very little affect on their binding affinity. Similarly, point mutations located relatively deep in TM III, VI, or VII strongly impaired the agonistic property of the nonpeptide compounds, again, without affecting the binding affinity of these compounds and, importantly, without affecting the ability of the peptide agonist angiotensin II to stimulate signal transduction. Thus, in both cases relatively small chemical modifications of either the ligand or the receptor affect the agonistic efficacy of these nonpeptide compounds dramatically, with only minor effects or no effect at all on their binding affinity.

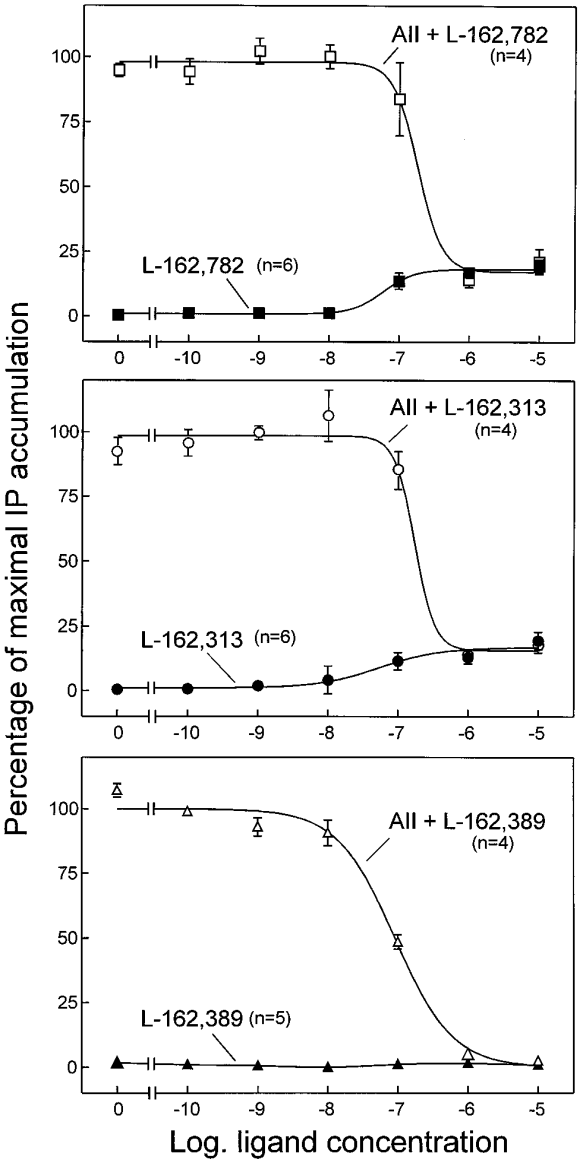

Fig. 7. Phosphatidylinositol (*IP*) turnover in response to nonpeptide compounds administered alone or with angiotensin II in transiently transfected COS-7 cells expressing the N295D rat AT_1 receptor. The phosphatidylinositol turnover is expressed as a percentage of the maximal response obtained during stimulation with angiotensin II stimulation [1.7 ± 0.2 fmol/min/10^5 cells (2100 cpm/10^5 cells); seven experiments; B_{max} = 40 ± 9 fmol/10^5 cells]. *Closed symbols*, dose-response curves for the three nonpeptide compounds when administered alone; *open symbols*, dose-response curves for the three nonpeptide compounds when given with angiotensin II ($10^{-8.5}$ M); *parentheses*, number of individual experiments performed in duplicate.

Partial agonism of nonpeptide compounds. A partial agonist is defined as a compound that produces submaximal responses and competitively blocks the effect of agonists of higher intrinsic efficacies (27). The AT_1 nonpeptide compounds of the current study are partial agonists, but instead of being competitive they are insurmountable antagonists (Fig. 3). This complicates the pharmacological analysis of their potency as antagonists because the Schild analysis is disturbed not only by the fact that they are partial agonists, which to a certain degree could be accounted for (27), but also by the fact that these compounds reduce the maximally achievable response (3).

The observations in the N295D rat AT_1 receptor may provide some insight into the mechanism of action of the nonpeptide compounds on the wild-type AT_1 receptor. This mutation does not affect the binding affinity of any of the three compounds, yet they all act as an almost pure antagonist in this receptor construct and, importantly, have IC_{50} values for inhibition of a submaximal stimulation with angiotensin that are similar to that of the most potent antagonist, L-162,389, on the wild-type receptor. These data indicate that the mutation has increased the antagonistic potency of the two other compounds to the same level as that of the L-162,389 compound. However, because no effect is observed on binding affinity, it could be suggested instead that possibly by impairing the ability of the receptor to mediate an agonistic response in conjunction with these compounds (through direct or indirect interaction), the mutation has revealed that the "default" pharmacological property of all three compounds when combined with the AT_1 receptor is antagonism. However, it should be emphasized that this obviously is pure speculation and is based on interpretations of the results and that this is not a model that is directly supported by the presented data. In the current study, we found a poor correlation between affinity and efficacy for the nonpeptide compounds in relation to both chemical modifications of the compounds and the receptor. It could be speculated that the receptor/ligand complex of these compounds after binding is able to change between inactive and active conformations, of which the latter is able to precipitate G protein activation. The presence of, for example, the "extra" methyl group in L-162,782 makes it possible for this compound to stabilize or induce an active conformation and thereby bias the equilibrium toward the active signaling conformation (more so than is the case for L-162,389). Interestingly, Arnis and Hofmann (28, 29) recently described a phenomenon in rhodopsin, in which an interchange between an active and an inactive conformation of the "cytoplasmic domain" of the molecule apparently can take place without changes in the isomerization of retinal, the bound "ligand," or in the retinal Schiff base located in the more exterior part of the membrane-spanning domain of this molecule. According to the recently published modification of the allosteric two-state model of receptor activation (30), the receptor undergoes transitions among a large population of allosteric states, all of which exist in either ligand bound or unbound form. For a ligand to be an agonist, the set of states stabilized by that ligand should include, but not necessarily be limited to, those conformations that are biologically active (30). This model excludes the otherwise tight correlation between affinity and efficacy implied by the classic two-state model (30). In relation to the current study, it could then be argued that the small chemical modification of either the ligand or the receptor, which makes the system less efficacious without changing the affinity, basically just shifts the set of conformations stabilized by the ligand toward one, which in total gives less activity.

Bell-shaped dose-response curves for the nonpeptide agonists. In general, several different mechanisms could account for bell-shaped dose-response curves. In tissue preparations, these curves could reflect a situation in which the compounds are able to activate both stimulatory and inhibitory receptor types at the same time (31–33). However, this cannot be the case in the current study, which was performed in tissue culture cells, either COS-7 cells or stable clones of transfected CHO cells expressing only one receptor type, the AT_1 (3). Our data do not provide an explanation for the bell-shaped curves. Although we observe only a relatively small nonspecific inhibitory effect of these compounds on phosphatidylinositol turnover induced by stimulation of the NK-1 receptor, and this occurs only at very high concentrations (10^{-5} M; data not shown), it cannot be excluded that such an nonspecific effect is responsible for part of the observed bell-shaped curve. It could obviously also be a reflection of some kind of complex postreceptor interaction. Another possible explanation is that the ligands are able to stabilize or induce both active and inactive conformations of the same receptor. Mechanistically, several models could be envisioned, of which the simplest one is a situation in which the binding site for the agonist is composed of two subsites that at high concentrations of ligand will each be occupied by a ligand molecule and thereby prevent the productive binding of a single molecule between the two half-sites. This mechanism seems to be the basis for the bell-shaped dose-response curve for, among others, growth hormone, in which the two half-sites are found on two separate receptor units (34).

Mutational mapping of binding sites for the nonpeptide compounds. In a library of mutant AT_1 receptors, some interesting differences were observed for the "agonistic" and the "antagonistic" compound in this series. Like the prototype biphenylimidazole antagonist L-158,809, the predominantly antagonistic compound L-162,389 was susceptible to a number of substitutions deep in the TMs, whereas the predominantly agonistic compound L-162,782 generally was unaffected by these substitutions. Nevertheless, one mutation, A104V, at the top of TM III impaired the binding of both compounds regardless of their biological properties. At the top of TM VII, an overlap in mutational susceptibility was found at residue Thr287 between angiotensin II and the new nonpeptide antagonist L-162,389. Interestingly, however, this was not the case for the structurally similar agonist L-162,782.

Surprisingly, we have not yet identified any receptor mutation that seriously affects the binding affinity of the prototype nonpeptide agonist L-162,313 (3) (Table 1), although some substitutions, such as N295D, certainly impair the ability of the compound to activate the receptor. One explanation for the relative resistance to mutational mapping of some compounds (e.g., the partial agonists of the current study) could be that these compounds are able to exploit more than one receptor conformation with almost equal affinity. In this scenario, certain residues could be especially important for the binding energy in one conformation and others could be especially important in the other conformation, although they all could be part of the same, general binding pocket. Point mutations would then have rather limited effects if they impair the binding to only one of the conformations, which would not be detected due to the almost normal binding to the other conformation of the receptor. Similarly, in the tachykinin NK-2 system, it has been very difficult to map the binding site for the insurmountable antagonist SR48,968 by point mutations. However, rather dramatic effects were observed when a couple of these "negative" point substitutions were combined (35). In the AT_1 system, we previously noted that insurmountable antagonists as a group were significantly less susceptible to receptor mutations than were

chemically similar competitive antagonists, although the structural reason for this remains unclear (14, 36).

Do the nonpeptide agonists mimic angiotensin II in their binding and action on the AT_1 receptor?. Unfortunately, this question cannot be answered on the basis of the current data, although the results tend to indicate major differences in their modes of action. However, it should be emphasized that although it is generally assumed that we are mapping binding sites by receptor mutagenesis, it is merely the susceptibility of the compounds to mutational exchange of receptor residues that is determined by this procedure (7). For example, it is nearly impossible to differentiate between effects caused by the exchange of actual contact residues and those caused by the exchange of second-row residues or residues located even farther in the receptor structure (i.e., substitutions that may indirectly affect the ligand binding) (7). Furthermore, certain mutations (e.g., V108A, H256A, and N295D of the current study) may affect the efficacy and not the affinity of ligands. It is uncertain whether such residues are necessarily part of the actual binding site for the ligand. If they are, they would then be part of a selective binding site for the nonpeptide agonist compounds located relatively deep between the TMs, in accordance with the previously identified general picture of the binding site for the nonpeptide compounds in this receptor system (9, 14, 37). Concerning angiotensin, a number of presumed contact residues have been identified mainly in the exterior part of the receptor and around the outer parts of the TMs (9–13, 37). As yet, we have been unable to identify any overlap between the presumed interaction points for nonpeptide agonist compounds and angiotensin (3) (Table 1). A possible common interaction site could be Lys199 in TM V because this residue apparently constitutes a crucial part of the presumed binding pocket for the peptide (10–13). Unfortunately, the low expression level of mutant receptors with substitutions at this position combined with the necessity for performing the binding assay with the nonpeptide compounds of this series under special conditions (e.g., in the absence of bovine serum albumin) has prevented us from probing this possibility. However, we did not find that the affinity of the L-162,782 and L-162,389 compounds was affected by substitution at position 199 when the binding assay was performed in the presence of bovine serum albumin.[3] Nevertheless, under these conditions, both compounds bound with an apparent low affinity to the wild-type receptor, which makes the interpretation of the results difficult. Importantly, the high affinity binding of both agonist and antagonist nonpeptide compounds of the new biphenylimidazole series was affected by the A104V substitution in TM III. This is an interesting position because in the molecular models, it is in close spatial proximity to the part of TM VII at which angiotensin seems to have major interaction points (Fig. 1 and Table 1) (9).

Importantly, the dramatic effect on receptor activation of the dualistic compounds caused by receptor substitutions such as V108A and N295D, which had minimal effect on their binding affinity and affected neither angiotensin binding nor coupling, emphasizes that functional data are very important in mutational analysis of ligand/receptor interactions.

[3] S. Perlman, H. T. Schambye, S. A. Hjorth, and T. W. Schwartz, unpublished observations.

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