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Site-Directed Mutagenesis of the Gerbil and Human Angiotensin II AT_1 Receptors Identifies Amino Acid Residues Attributable to the Binding Affinity for the Nonpeptidic Antagonist Losartan

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ABSTRACT

Gerbil angiotensin II AT_1 receptors have more than 90% amino acid sequence homology with human AT_1 receptors and similar affinity for the natural peptide agonist angiotensin II. However, their binding affinity for the biphenylimidazole AT_1 receptor antagonist losartan is greatly reduced compared with the hAT_1 receptor (400 times lower for the gAT_{1A} receptor and 40 times lower for the gAT_{1B} receptor cloned here). Gain- and loss-of-function site-directed mutagenesis revealed that in gerbil and human AT_1 receptors, the amino acid most important for losartan binding is located in position 108, followed by 107, both in transmembrane (TM) III. In both gerbil and human AT_1 receptors, the effect of G107S and I108V mutants is cumulative. Mutation L195M in TM V is very important, when combined with mutations G107S and I108V, for both gerbil and human AT_1 receptors. In the gerbil, less important amino acids are located in positions 150/151 (TM IV) and 177 in the extracellular loop 2. The study of gerbil natural mutants allowed us to advance our understanding of amino acids selectively involved in the determination of antagonist affinity for gerbil and, most importantly, for human angiotensin II AT_1 receptors.

Angiotensin II (Ang II) is a circulating and local tissue hormone regulating fluid and electrolyte metabolism, hormone secretion, the autonomic nervous system, and brain function (Saavedra, 1992; Matsusaka and Ichikawa, 1997; Timmermans, 1999). There are two types of mammalian Ang II receptors, AT_1 and AT_2. AT_1 receptors are selectively antagonized by nonpeptidic biphenylamidazoles or by imidazoleacrylic compounds (Timmermans, 1999). Stimulation of AT_1 receptors modulates Ang II effects, and nonpeptide AT_1 receptor antagonists are used in hypertension treatment (Timmermans, 1999).

Ang II AT_1 receptors belong to the seven-transmembrane G-protein–coupled receptor superfamily (Sandberg, 1994). Mammalian AT_1 receptors have higher than 90% amino acid sequence identity and similar affinity for peptide ligands, such as Ang II. Many mammalian AT_1 receptors express an affinity for the biphenylamidazole antagonist losartan in the same range as human AT_1 receptors (Chiu et al., 1993), but losartan affinity is reduced in bovine, canine, ferret, and porcine AT_1 receptors (Chiu et al., 1993; Itazaki et al., 1993; Burns et al., 1994; Gosselin et al., 2000). Amphibian Ang II receptors, with lower homology to mammalian AT_1 receptors, also bind peptide ligands with similar affinity (Sandberg, 1994) but have affinity for losartan several orders of magnitude lower that that of mammalian receptors (Ji et al., 1995). This suggests that the nonpeptide binding domain was largely distinct from the receptor domain involved in Ang II binding (De Gasparo et al., 2000). Such differences between domains involved in the recognition of peptide and nonpeptide ligands hold true for many other G-protein–coupled receptors (Beinborn et al., 1993; Gether et al., 1993; Kong et al., 1994).

For AT_1 receptors, important epitopes involved in Ang II binding may be located around the top of transmembrane segments I, II, and VII, in close spatial proximity in the folded receptor structure (Hjorth et al., 1994). Binding of nonpeptide Ang II antagonists may be dependent on nonconserved residues located deep in the hydrophobic transmembrane segments of the AT_1 receptor, as demonstrated by mutational analysis of the mammalian AT_1 and amphibian Ang II receptors (Bihoreau et al., 1993; Ji et al., 1993, 1994, 1995; Marie et al., 1994; Schambye et al., 1994; Noda et al., 1995; Monnot et al., 1996; Inoue et al., 1997; De Gasparo et al., 2000).

In rats and mice, there are two AT_1 receptor subtypes (AT_{1A} and AT_{1B}) encoded by different genes and with significant sequence homology in the coding regions [open-reading frames (ORF)] (Iwai and Inagami, 1992; Sasamura et al.,

ABBREVIATIONS: Ang II, angiotensin II; Sar[1]-Ang II, sarcosine[1]-angiotensin II; TM, transmembrane; ORF, open-reading frames; HBSS, Hanks' balanced salt solution; UTR, untranslated region; bp, base pair(s).

1992; Yoshida et al., 1992). AT_{1A} and AT_{1B} receptors have similar affinity for nonpeptide receptor antagonists and cannot be differentiated pharmacologically, although they are differentially localized and regulated (Iwai and Inagami, 1992; Sasamura et al., 1992; Yoshida et al., 1992).

In another rodent species, the gerbil, we found an Ang II receptor subtype that had high affinity for Ang II but was unable to recognize nonpeptide antagonists (De Oliveira et al., 1995). Cloning the receptor from a gerbil kidney cDNA library revealed higher than 90% homology to mammalian AT_1 receptors and a difference from the hAT_1 receptor at only 25 amino acid residues (Moriuchi et al., 1998). The receptor expressed high affinity for Ang II, similar to the human AT_1 receptor, but greatly reduced (400-fold) affinity for losartan (Moriuchi et al., 1998). This gerbil receptor had a distribution similar to other rodent AT_{1A} receptors, with closer homology to rodent (rAT_{1A} and mAT_{1A}) receptors than to their AT_{1B} subtypes (Moriuchi et al., 1998). We considered this gerbil AT_1 receptor as a gAT_{1A} subtype (Moriuchi et al., 1998).

Gerbils transcribed an additional AT_1 receptor subtype that had affinity for Ang II similar to that of the hAT_1 receptor but an affinity for losartan intermediate between that of the hAT_1 and the gAT_{1A} receptor. This receptor was specifically localized to the adrenal zona glomerulosa, with an affinity for losartan 40-fold lower than that of the hAT_1 receptor (Moriuchi et al., 1998). We speculated that the second gerbil AT_1 receptor was in fact the rodent AT_{1B} subtype.

We initiated studies to identify the amino acid residues responsible for the reduced affinity to nonpeptide antagonists of the naturally mutated gAT_1 receptors. This presented a distinct advantage, because natural mutants probably keep the whole integrity of the three-dimensional structure for Ang II binding without major distortions. Site-directed mutagenesis of gerbil and human AT_1 receptors, with a combination of gain- and loss-of-function experiments, could further advance the identification of amino acids involved in the binding mechanism of nonpeptide antagonists.

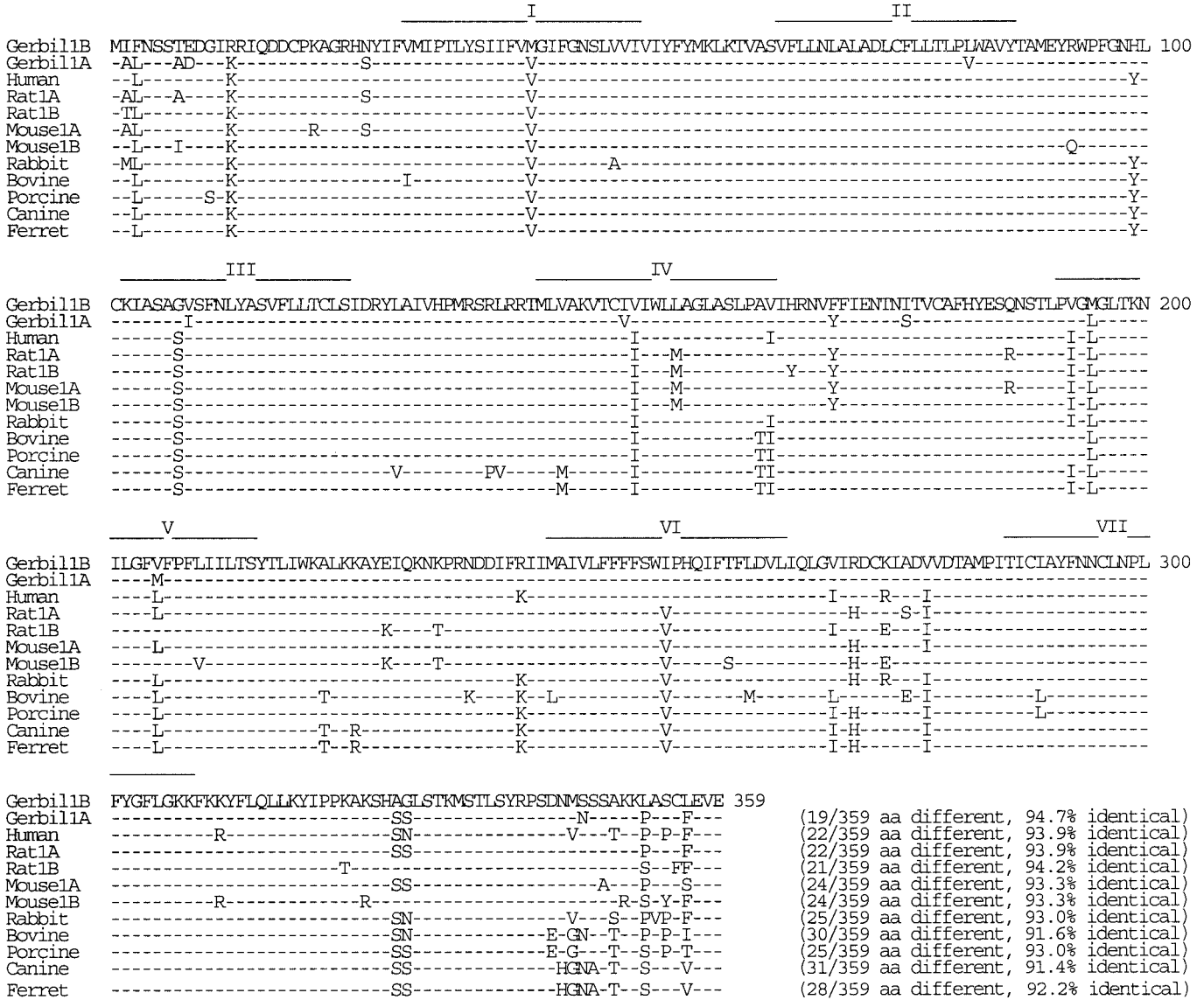

Fig. 1. Alignment of the gerbil angiotensin II AT_{1B} receptor amino acid sequence with those of other mammalian AT_1 receptors. -represents identical amino acids. Amino acid differences are shown in their corresponding positions. Solid lines indicate prospective transmembrane domains I through VII. Amino acid positions are numbered on the right. The gerbil AT_{1B} nucleotide sequence was deposited in GenBank (accession number AF 078794).

Experimental Procedures

Animals. We purchased male Mongolian gerbils (*Meriones unguiculatus*, 65–80 g) from Tumblebrook Farm (West Brookfield, MA). The animals were kept under standard conditions with food and water ad libitum and were killed by decapitation between 9:00 and 10:00 AM. To study receptor binding and in situ hybridization, adrenal glands and kidneys were dissected and frozen in isopentane on dry ice. Tissue sections (16-μm thickness) were cut in a cryostat and kept at −80°C until used. The National Institutes of Health Animal Care and Use Committee approved all animal procedures.

Materials. ^{125}I-Sar^1-Ang II (specific activity, 2200 Ci/mmol) and RNA labeling kits were purchased from PerkinElmer Life Sciences (Boston, MA). Ang II was purchased from Peninsula Laboratories (Belmont, CA). ^{35}S-UTP (specific activity, >1000 Ci/mmol) and Hyperfilm-^{3}H were obtained from Amersham Biosciences (Piscataway, NJ). Losartan was a gift from DuPont Merck Pharmaceutical Co. (Wilmington, DE). Cell culture products (Opti-MEM and Dulbecco's modified Eagle's medium) and binding buffers [Hanks' balanced salt solution (HBSS)] were purchased from Invitrogen (Carlsbad, CA). The monkey kidney epithelial COS-7 cell line was obtained from American Type Culture Collection (Manassas, VA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The cDNA synthesis kit, DNA labeling kit (Prime-It II), and QuikChange site-directed mutagenesis kit were obtained from Stratagene (La Jolla, CA). The pcDNA3.1 vector was purchased from Invitrogen. Nucleotide sequence analysis was performed using an ABI Prism 310 sequencing machine and a Big Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The vector plasmid with the hAT_1 receptor cDNA insert was a generous gift from Dr. T. Inagami (Vanderbilt University School of Medicine, Nashville, TN).

Molecular Cloning of gAT_{1B} Receptor Gene. We constructed a cDNA library from gerbil adrenal gland mRNA and selected a cDNA insert in the range of about 1.8 to 4 kb. A complexity of 1×10^6 independent clones was constructed in a λ uni-ZAP vector. Independent clones were screened with a conventional in situ plaque hybridization method, using hAT_1 receptor ORF cDNA as a probe, and the positive clones were screened and hybridized again to a probe directed to the gAT_{1A} 3′-UTR. Selected clones containing the gAT_{1B} receptor gene were treated to make in vivo conversion from the λ uni-ZAP vector into a phagemid vector, pBK-CMV, and were subject to further nucleotide sequence analysis using an ABI Prism 310 sequencing machine (Applied Biosystems).

In Situ Hybridization. A specific riboprobe directed to the 3′-UTR of the cloned gAT_{1B} receptor was obtained after subcloning of a polymerase chain reaction-generated DNA fragment of 729 bp into the *Xba*I-*Eco*RI site of the pBluescript II KS$^-$ vector (Stratagene). The DNA fragment was amplified with *Xba*I-extended forward and *Eco*RI-containing reverse primers, corresponding to nucleotides 1128 through 1856. Antisense and sense (control) riboprobes were labeled by in vitro transcription in the presence of 200 μCi of ^{35}S-UTP (Amersham Biosciences; >1000 Ci/mmol). In situ hybridization was performed as described previously (Moriuchi et al., 1998), with modifications as follows: adrenal gland and kidney sections were covered with hybridization buffer containing 4×10^4 cpm/μl of probe, hybridized for 18 h at 54°C, treated with RNase A, and washed with increasing stringency. After a final high-stringency wash in 0.1 × standard saline citrate at 65°C for 1 h, sections were dehydrated, exposed to Hyperfilm-^{3}H for 4 days, and developed as described previously (Moriuchi et al., 1998).

Expression of Angiotensin II Receptors in COS-7 Cells. Monkey kidney epithelial COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l of glucose, 4 mM glutamine, 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. One day after plating in tissue culture plates (2×10^6 cells/100 cm^2), cells were washed with 10 ml of Opti-MEM. The gAT_{1A}, gAT_{1B}, and hAT_1 receptors and receptor chimera cDNAs were subcloned into the pcDNA3.1 vector. Four micrograms of vector DNA in 800 μl of Opti-MEM, and 16 μl of LipofectAMINE (Invitrogen) in 800 μl of Opti-MEM, were mixed and incubated at room temperature for 30 min. The DNA/LipofectAMINE complex was added to each plate after mixing with 6.4 ml of Opti-MEM. The next day, transfected cells were divided into wells of 24-well tissue culture plates (1×10^5 cells/well).

Ligand Binding and Displacement Study. The ligand displacement analysis was performed the day after the split of the transfected cells into 24-well plates. Cells were washed with binding buffer (0.2% bovine serum albumin in HBSS) and incubated at room temperature for 2 h with 0.3 nM ^{125}I-Sar^1-Ang II with variable concentrations (10^{-12} to 10^{-3} M) of the unlabeled competitive peptide agonist (Ang II) or the selective nonpeptidic AT_1 receptor antagonist losartan. After 2 h of incubation, the cells were washed with ice-cold HBSS three times, dissolved in 0.2 M NaOH, and bound ^{125}I-Sar^1-Ang II was measured in a gamma counter. Each experi-

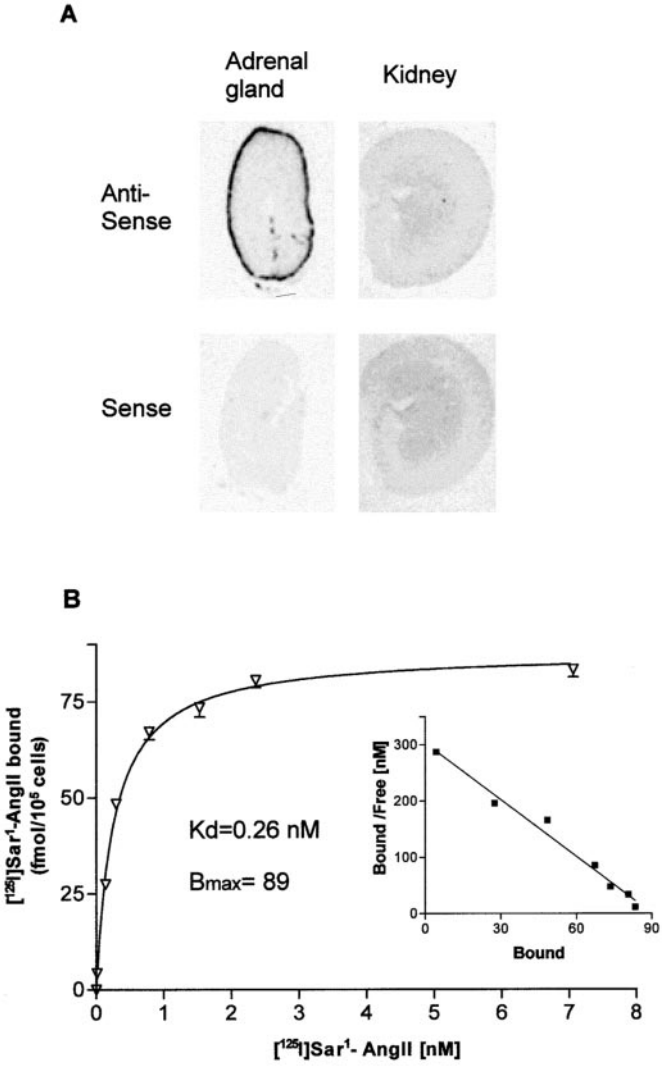

Fig. 2. A, autoradiograms of gerbil adrenal gland and kidney. Figures represent in situ hybridization with antisense or sense probes specific for the 3′-untranslated region of the cloned gerbil angiotensin II AT_{1B} receptor cDNA. Note the positive signal in the gerbil adrenal zona glomerulosa and the absence of signal in the gerbil kidney with the antisense riboprobe. B, saturation isotherm of specific ^{125}I-Sar^1-Ang II binding to the gerbil angiotensin II AT_{1B} receptor transfected to COS-7 cells. Insert shows a Scatchard plot. The figure represents a typical experiment repeated three times in triplicate.

TABLE 1

Gain-of-function mutations in gAT_{1A} receptors

Data are the mean ± S.E.M. obtained from two to four experiments (each done in triplicate) after displacement of ^{125}I-Sar^1-Ang II binding by increasing concentrations of losartan, for selected gAT_{1A} mutant receptors transfected to COS-7 cells. To make F_{mut} values comparable, the F_{mut} values of each mutant used the human EC_{50} value as a control for comparison.

Receptor	Ang II		Losartan	
	EC_{50}	F_{mut}	EC_{50}	F_{mut}
	nM		*nM*	
Wild-type				
hAT_1	4.5 ± 0.6	1.0	15 ± 2.4	1.0
gAT_{1A}	5.4 ± 0.7	1.2	5900 ± 870	393
gAT_{1A} variants: gain-of-function				
TMII				
V83L	6.0 ± 1.0	1.3	4980 ± 440	332
TMIII				
G107S/I108V	4.7 ± 0.6	1.0	20 ± 3.2	1.3
G107S	5.0 ± 0.4	1.1	610 ± 75	41
I108V	5.3 ± 0.4	1.2	101 ± 14	6.7
TMIV				
V150I/V151I	3.8 ± 0.6	0.8	2950 ± 290	197
Extracellular loop 2				
S177I	3.0 ± 0.5	0.7	3160 ± 330	211

F_{mut}, mutant EC_{50}/hAT_1 EC_{50}.

ment was carried out at least twice in triplicate. The binding data were analyzed, and EC_{50} values were determined by computerized nonlinear regression analysis using GraphPad Prism 2.0 software (GraphPad Software, San Diego, CA). To compare relative affinities of multiple mutants, we compared them with the affinity of the wild-type hAT_1 receptor. We determined F_{mut} values as the ratio of mutant EC_{50}/hAT_1 EC_{50} values.

Representation of the Presumed Binding Pocket for the Biphenylimidazole Antagonists. The sequence of the hAT_1 receptor was analyzed using the SOSUI system software (version 1.0) (Hirokawa et al., 1998) to obtain a two-dimensional representation (Schambye et al., 1994) and a helical wheel diagram (Murgolo et al., 1996). Positions considered important for losartan binding were identified based on the present results and those of the literature.

Results

Molecular Cloning of the New Gerbil AT_1 Receptor. We constructed a cDNA library from gerbil adrenal gland, selected inserts in the range of 1.8 to 4 kb, and constructed a complexity of 1×10^6 clones in a γ-uni-ZAP vector. We screened approximately 80,000 independent clones by conventional in situ plaque hybridization methods, using human AT_1 receptor ORF cDNA as a probe; we identified 19 positive clones. To exclude clones positive for gAT_{1A}, the positive clones were screened and hybridized again to a probe directed to the gAT_{1A} 3′-UTR. None of the 19 positive clones hybridized to this probe. Four different clones, designated as 3, 4, 6, and 9, were finally selected and thoroughly sequenced. The nucleotide sequence of the longest clone (clone 4; 2,745 bp), contained an ORF of 1,077 bp encoding a protein of 359 amino acid residues with a 3′-UTR 700 bp longer than that present in the other clones. Clone 9 had a 3′-UTR shorter than that of clone 4 but an extra 96-bp exon in 5′-UTR. Clones 3 and 6 had 3′-UTRs of similar length as that of clone 9, with the exception of the extra exon. The molecular characteristics of the cloned gerbil cDNA were similar to those of other mammalian AT_1 receptors (Moriuchi et al., 1998).

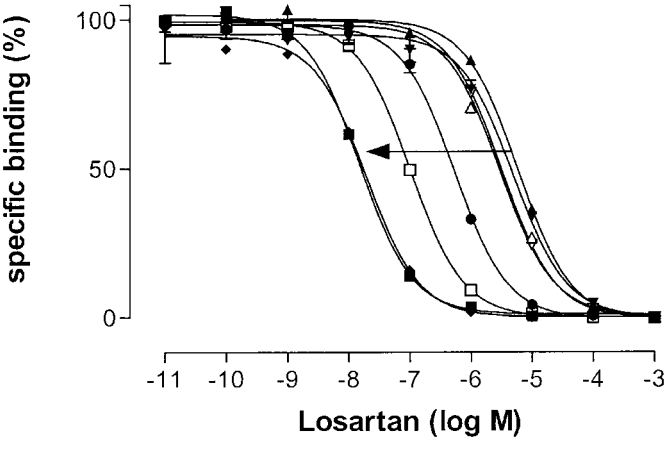

Fig. 3. Gain-of-function mutations in gerbil AT_{1A} receptor. The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected gAT_{1A} mutant receptors transfected to COS-7 cells. The arrow shows the progressive gain of function compared with the hAT_1 receptor. ■, hAT_1; ▲, gAT_{1A}; ▼, V83L; ♦, G107S/I108V; ●, G107S; □, I108V; △, V150I/V151I; ▽, S177I.

Sequence comparison of amino acids between the novel gAT_1 receptor and hAT_1 receptors revealed a 93.9% identity, with only 22 different residues (Fig. 1). Two mismatches were found in the amino terminal extracellular region, and eight mismatches were found localized to the carboxyl-terminal intracellular tail. The three intracellular loops were very well conserved, with only one amino acid difference, located in the third loop (Arg^{240}). Five different amino acid residues were found in the three extracellular loops (His^{99} in the first, Val^{193} in the second, and Val^{270}, Lys^{275}, and Val^{279} in the third loops). Six different amino acid residues were located in the transmembrane domains (Met^{41} in TM I; Gly^{107} in TM III; Val^{151} and Val^{164} in TM IV; and Met^{195} and Val^{205} in TM V) (Fig. 1). The sequence homology of the novel gAT_1 receptor was similar to that of the rat and mouse AT_{1B} receptors (Asn^{25} and not Ser^{25}, Gln^{187} and not Arg^{187}, Ala^{328} and not Ser^{328}, and Gly^{329} and not Ser^{329}).

In situ hybridization with the use of a specific riboprobe directed to the 3′-UTR of the newly cloned gAT_1 receptor revealed receptor mRNA in the adrenal zona glomerulosa and not in the adrenal medulla or the kidney (Fig. 2A). After expression in COS-7 cells, the cloned receptor bound the

TABLE 2

Gain-of-function multiple combinatorial mutations in gAT_{1A} receptors

Data are the mean ± S.E.M. obtained from two to four experiments (each done in triplicate) after displacement of ^{125}I-Sar^1-Ang II binding. by increasing concentrations of losartan, for selected gAT_{1A} mutant receptors transfected to COS-7 cells. To make F_{mut} values comparable, the F_{mut} values of each mutant used the human EC_{50} value as a control for comparison.

Receptor	Ang II		Losartan	
	EC_{50}	F_{mut}	EC_{50}	F_{mut}
	nM		*nM*	
Wild-type				
hAT_1	4.5 ± 0.6	1.0	15 ± 2.4	1.0
gAT_{1A}	5.4 ± 1.2	1.2	5900 ± 870	393
gAT_{1A} variants: gain-of-function				
G107S/I108V/V150I/V151I	3.9 ± 0.3	0.9	16 ± 3.0	1.1
G107S/I108V/S177I	4.0 ± 0.2	0.9	25 ± 2.4	1.7
G107S/I108V/V150I/V151I/S177I	4.2 ± 0.4	0.9	29 ± 4.1	1.9
G107S/I108V/V150I/V151I/S177I/V83L	6.1 ± 0.3	1.4	40 ± 3.7	2.7
V150I/V151I/S177I	3.3 ± 0.6	0.7	2750 ± 340	183
V150I/V151I/S177I/V83L	4.7 ± 0.7	1.0	8300 ± 990	553

F_{mut}, mutant EC_{50}/hAT_1 EC_{50}.

peptide agonist ^{125}I-Sar^1-Ang II with high affinity and in a concentration-dependent and saturable manner (Fig. 2B). Conversely, this receptor showed a greatly reduced binding affinity for the antagonist biphenylimidazole derivative losartan, 40 times lower compared with that of the hAT_1 receptor (Table 3; Fig. 5, A and B). Because of its sequence homology and selective localization, similar to those of other rodent AT_{1B} receptors, we considered the novel receptor as the gAT_{1B} subtype.

Selection of Mutagenesis Sites. Our initial studies with gAT_{1A} and hAT_1 chimerical receptors identified the responsible region for the reduced affinity for losartan as located between the TM II and TM VII helices (amino acids 63–321) (R. Moriuchi and J. M. Saavedra, unpublished observations). Eight of the amino acids in the gAT_{1A} receptor that are different from the hAT_1 receptor were identified in this region (Val^{83} in TM II; Gly^{107} and Ile^{108} in TM III; Val^{150}, Val^{151}, and Val ^{164} in TM IV; Ser^{177} in the extracellular loop 2; and Met^{205} in TM V) replacing Leu^{83}, Ser^{107}, Val^{108}, Ile^{150}, Ile^{151}, Ile^{164} Ile^{177}, and Leu^{205} in hAT_1 receptors (Moriuchi et al., 1998).

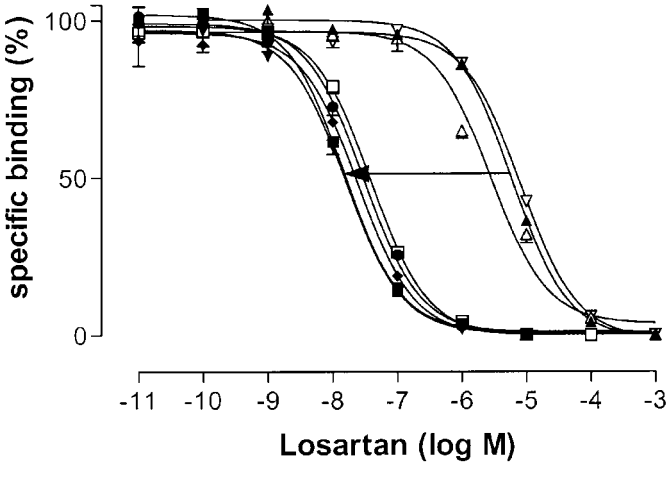

Fig. 4. Gain-of-function multiple combinatorial mutations in gerbil AT_{1A} receptor. The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected gAT_{1A} mutant receptors transfected to COS-7 cells. The arrow shows the progressive gain of function compared with the hAT_1 receptor. ■, hAT_1; ▲, gAT_{1A}; ▼, G107S/I108V/V150I; ♦, G107S/I108V/S177I; ●, G107S/I108V/V150I/V151I/S177I; □, G107S/I108V/V150I/V151I/S177I/V83L; △, V150I/V151I/S177I; ▽, V150I/V151I/S177I/V83L.

The newly cloned gAT_{1B} receptor had five amino acids different from the hAT_1 receptor in the TMII/TMVII domain (Gly^{107} in TM III; Val^{151} and Val^{164} in TM IV; and Met^{195} and Val^{205} in TM V), replacing Ser^{107}, Iso^{151}, Iso ^{164}, Leu^{195}, and Leu^{205} in hAT_1 receptors. Thus, Ile^{108} and Val^{150}, mutated in the gAT_{1A}, were conserved in the gAT_{1B} compared with the hAT_1 receptor. Conversely, Met^{195} was mutated in the gAT_{1B} receptor and conserved in the gAT_{1A} receptor compared with the hAT_1 receptor.

Based on sequence comparison between cloned mammalian AT_1 receptors, we selected six amino acids (Val^{83}, Gly^{107}, Ile^{108}, Iso^{150}, Iso^{151}, and Iso^{177}) and three amino acids (Gly^{107}, Val^{151}, and Met^{195}) as major targeting sites on gAT_{1A} and gAT_{1B} receptor subtypes, respectively, for our site-directed mutagenesis study. Based on preliminary experiments, we selected amino acids in the most important positions (Gly^{107}, Ile^{108}, and Met^{195}) for complementary loss-of-function mutations in the hAT_1 receptor.

Gain-of-Function Mutation in gAT_{1A} Receptors. We performed gain-of-function assays with gAT_{1A} mutants in single individual positions followed by the analysis of dual combinatorial mutants. Single-mutant analysis revealed substantial gain of function for mutants in both positions 107 and 108. The replacement of I108V was six times more effective than G107S (Table 1; Figure 3). The combinatorial replacement with G107S/I108V resulted in a binding affinity for losartan that was comparable with that of the wild-type hAT_1.

There was a much lower gain of function after other mutations on individual positions of the gAT_{1A} receptor. The variants of V150I/V151I and S177I improved losartan-binding affinity only by two times, and the variant of V83L did not show any significant improvement for losartan binding (Table 1; Fig. 3). The rank of order for losartan-binding affinity was hAT_1 = (G107S/I108V) < I108V < G107S < (V150I/V151I) = S177I < V83L = gAT_{1A} (Table 1). Thus, Gly^{107} and Ile^{108} in TM III are attributable mainly to the reduced losartan binding of the gAT_{1A} receptor.

Combinatorial Gain-of-Function Mutations in Gerbil AT_{1A}. The combinatorial variant (G107S/I108V/V150I/V151I) obtained by addition of mutations V150I/V151I to the

TABLE 3

Gain-of-function mutations in gAT_{1B} receptors

Data are the mean ± S.E.M. obtained from two to four experiments (each done in triplicate) after displacement of ^{125}I-Sar^1-Ang II binding. by increasing concentrations of losartan, for selected gAT_{1B} mutant receptors transfected to COS-7 cells. To make F_{mut} values comparable, the F_{mut} values of each mutant used the human EC_{50} value as a control for comparison.

Receptor	Ang II		Losartan	
	EC_{50}	F_{mut}	EC_{50}	F_{mut}
	nM		*nM*	
Wild-type				
hAT_1	4.5 ± 0.6	1.0	15 ± 2.4	1.0
gAT_{1B}	3.3 ± 0.4	0.7	567 ± 54	38
gAT_{1B} variants: gain-of-function				
G107S	4.1 ± 0.4	0.9	192 ± 21	13
V151I	2.4 ± 0.5	0.5	557 ± 69	37
M195L	6.6 ± 0.9	1.5	155 ± 19	10
G107S/V151I	2.9 ± 0.5	0.6	156 ± 30	10.4
G107S/M195L	4.3 ± 0.4	1.0	43 ± 2.5	2.9

F_{mut}, mutant EC_{50}/hAT_1 EC_{50}.

mutants G107S/I108V recovered its losartan-binding affinity to a value very similar to that of the hAT_1 wild-type receptor (F_{mut} = 1.1) (Table 2; Fig. 4), but the effect was not different from that of the mutants G107S/I108V alone (Table 1).

The combinations with mutants that, when studied individually, improved losartan-binding affinity, such as S177I into G107S/I108V, were slightly detrimental. However, when S177I was added into the V150I/V151I mutant, we could not find any detrimental effect. Similarly, we found a slightly detrimental effect when the mutant S177I was added to the combinatorial G107S/I108V/V151I/V151I (Table 2; Fig. 4).

We found a similar phenomenon with the V83L mutant. When this mutant was added to any mutant combinations G107S/I108V/V150I/V151I/S177I or to V150I/V151I/S177I/V83L, it showed detrimental effects (Table 2; Fig. 4).

Our results indicated the following rank order for combinatorial mutants on losartan-binding affinity: hAT_1 = (G107S/I108V/V150I/V151I) < (G107S/I108V/S177I) < (G107S/I108V/V150I/V151I/S177I) < (G107S/I108V/V150I/151I/S177I/V83L) ≪ (V150I/V151I/S177I) < gAT_{1A} < (V150I/V151I/S177I/V83L) (Table 2).

Gain-of-Function Mutation in gAT_{1B} Receptors. Our experiments revealed that single amino acid replacement (G107S or M195L) resulted in a 3- to 4-fold gain of function as evidenced by the increase in losartan-binding affinity (Table 3; Fig. 5A). The V151I mutant did not produce a detectable gain of function (Table 3; Fig. 5A). When the mutants G107S and/or M195L were combined, the gain of function increased to 13-fold, indicating that the contribution of these two sites is exclusive and additive (Table 3; Fig. 5B). However, the gain of function produced by the G107S/M195L mutant was incomplete, and the binding affinity for losartan was still 3-fold lower compared with the wild-type gAT_{1B} receptor (Table 3; Fig. 5B). As expected, the double mutant G107S/V151I exhibited a gain of function similar to that of the single G107S mutant (Table 3; Fig. 5, A and B). We obtained the following rank order for combinatorial mutants on losartan-binding affinity: hAT_1 < G107S/M195L < M195L = G107S/V151I = G107S < V151I = gAT_{1B} (Table 3).

Loss-of-Function Mutation in gAT_{1A} and gAT_{1B} Receptors. To confirm the role of the amino acid in position 195, we constructed a L195M mutant on the gAT_{1A} receptor. The wild-type gAT_{1A} receptor exhibits a 400-fold lower binding affinity for losartan while retaining a conserved amino acid (Leu) in position 195. We found that the gAT_{1A} L195M

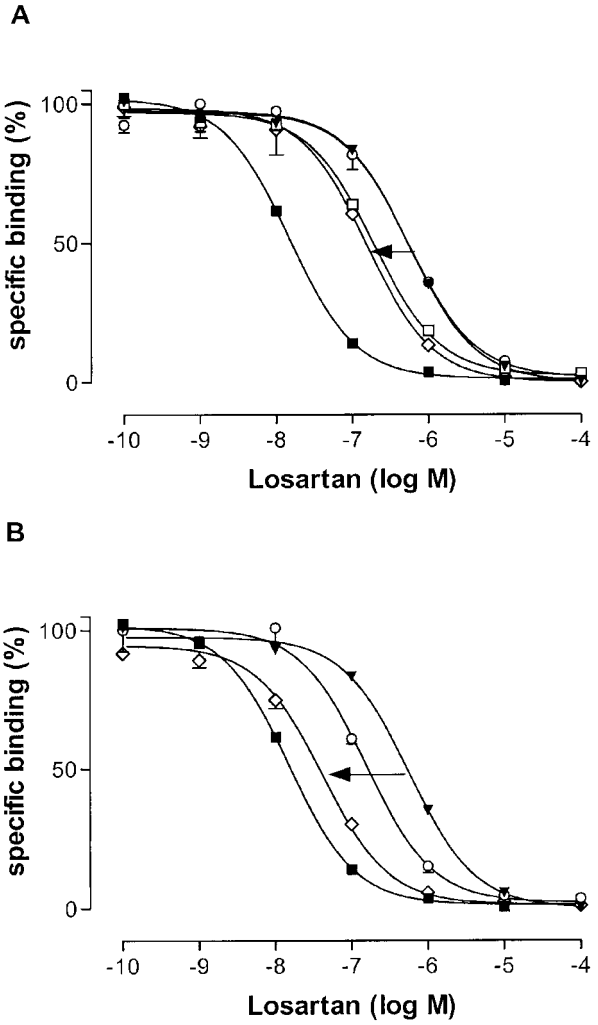

Fig. 5. A, gain of function in gerbil AT_{1B} receptor after single mutations. The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected gAT_{1B} mutant receptors transfected to COS-7 cells. The arrow shows the progressive gain of function compared with the hAT_1 receptor. ■, hAT_1; ▼, gAT_{1B}; □, G107S; ○, V151I; ◇, M195L. B, gain of function in gerbil AT_{1B} receptor after combinatorial mutations. The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected gAT_{1B} mutant receptors transfected to COS-7 cells. The arrow shows the progressive gain of function compared with the hAT_1 receptor. ■, hAT_1; ▼, gAT_{1B}; ○, G107S/V151I; ◇, G107S/M195L.

TABLE 4

Loss-of-function mutations in gAT_{1A} and gAT_{1B} receptors

Data are the mean ± S.E.M. obtained from two to four experiments (each done in triplicate) after displacement of ^{125}I-Sar^1-Ang II binding by increasing concentrations of losartan, for selected AT_{1A} and gAT_{1B} mutant receptors transfected to COS-7 cells. To make F_{mut} values comparable, the F_{mut} values of each mutant used the human EC_{50} value as a control for comparison.

Receptor	Ang II		Losartan	
	EC_{50}	F_{mut}	EC_{50}	F_{mut}
	nM		*nM*	
Wild-type				
hAT_1	4.5 ± 0.6	1.0	15 ± 2.4	1.0
gAT_{1A}	5.4 ± 0.7	1.2	5,900 ± 870	393
gAT_{1B}	3.3 ± 0.4	0.7	567 ± 54	38
gAT_{1A} mutations: loss-of-function				
gAT_{1A}/L195M	7.5 ± 1.1	1.4	20,830 ± 2,800	1,389
gAT_{1B} mutations: loss-of-function				
gAT_{1B}/V108I	6.7 ± 1.8	1.5	28,200 ± 5,600	1,880

F_{mut}, mutant EC_{50}/hAT_1 EC_{50}.

mutant showed an additional 4-fold decrease in losartan binding (F_{mut} = 1389; Table 4; Fig. 6A).

The wild-type gAT_{1B} receptor exhibits a 40-fold lower binding activity for losartan while retaining a conserved amino acid (Val) in position 108. We introduced Ile in replacement of Val^{108} into the gAT_{1B} receptor. The variant gAT_{1B} with the exogenous V108I mutation revealed a much lower binding affinity for losartan (F_{mut} = 1880) than that of wild-type gAT_{1A} (F_{mut} = 393) and a 40-fold lower affinity for losartan than that of the wild-type gAT_{1B} (Table 4; Fig. 6B). These experiments reveal that, in the gerbil, the effects of G107S, I108V, and M195L are cumulative. We obtained the following rank order for loss-of-function mutations in the gAT_{1A} and gAT_{1B} receptors: $hAT_1 < gAT_{1B} < gAT_{1A} \ll gAT_{1A}$/L195M < gAT_{1B}/V108I (Table 4).

Mutagenesis of hAT_1 Receptors with Loss-of-Function. To confirm the role of amino acids in positions 107, 108, and 195, we constructed hAT_1 mutants and determined the possibility of loss of function. The S107G mutant produced a 2.7-fold decrease in losartan-binding, whereas the V108I mutant decreased losartan-binding affinity 45-fold (Table 5; Fig. 8A). The double mutant S107G/V108I resulted in a more pronounced, 160-fold loss of function (Table 5; Fig. 7A).

Conversely, the single L195M mutant did not show any significant change in losartan-binding affinity (Table 5; Fig. 8B). Surprisingly, the double mutants L195M/S107G and V108L/L195M reduced losartan affinity 7.3-and 79-fold, respectively (Table 5; Fig. 7B). Moreover, the combinatorial mutant S107G/V108I/L195M decreased binding affinity by 420-fold (Table 5; Fig. 7B). These experiments confirm that, in the hAT_1 receptor, amino acids in positions 107, 108, and 195 are important. The role of position 195 is only revealed when amino acids in positions 107 or 108 are mutated, and the effect is proportionally higher when the mutants are combined. We obtained the following rank order for loss-of-function mutations in the hAT_1 receptor: hAT_1 = L195M < S107G < S107G/L195M < gAT_{1B} = V108I < V108L/L195M < S107G/V108I < gAT_{1A} = S107G/V108I/L195M (Table 5).

Mutations at Position Ile^{108}. We compared the effects of different amino acids in position 108, the most important for losartan-binding affinity. The V108I mutant in the hAT_1 receptor decreased binding affinity 45-fold (Table 6; Fig. 8), whereas in the gAT_{1A}, with the natural mutant V108I, binding affinity was about 400-fold lower than in the hAT_1 recep-

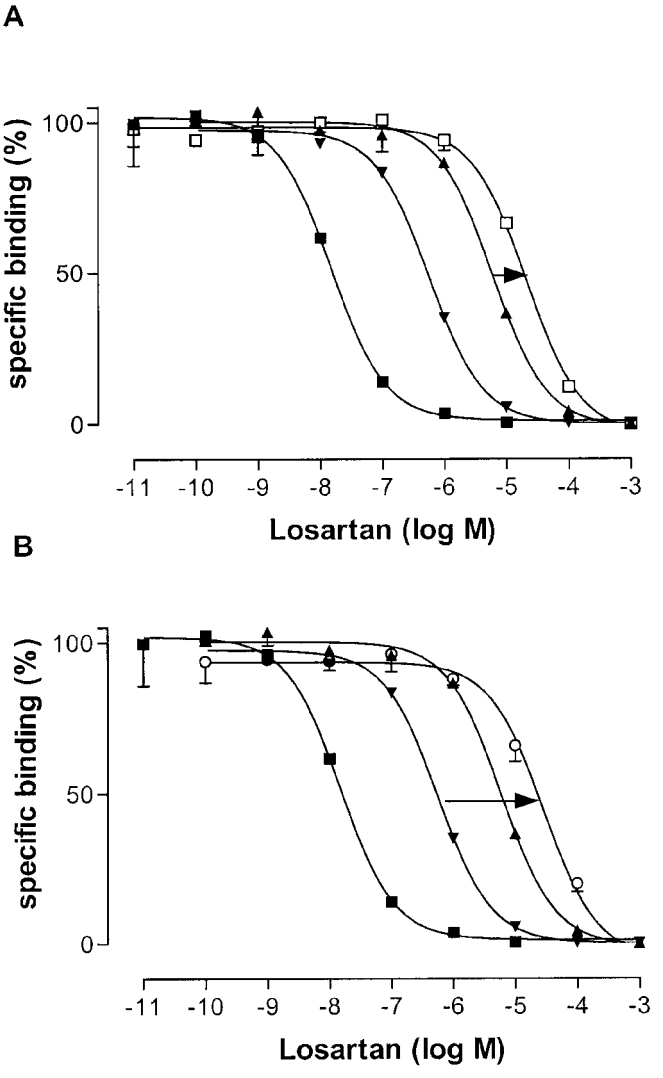

Fig. 6. A, loss-of-function mutations in gerbil AT_{1A} receptors. The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected gAT_{1A} mutant receptors transfected to COS-7 cells. The arrow shows the progressive loss of function compared with the hAT_1 receptor. ■, hAT_1; ▲, gAT_{1A}; ▼, gAT_{1B}; □, gAT_{1A}+L195M. B, loss-of-function mutations in gerbil AT_{1B} receptors. The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected gAT_{1B} mutant receptors transfected to COS-7 cells. The arrow shows the progressive loss of function compared with the hAT_1 receptor. ■, hAT_1; ▲, gAT_{1A}; ▼, gAT_{1B}; ○, gAT_{1B}+V108I.

tor (Table 6; Fig. 8). In the gAT_{1A} receptor, the mutant I108V produced a significant gain of function and the mutant I108A resulted in a gain of function of a lesser degree (Table 6; Fig. 8). The Ala residue was better than the Ile residue but detrimental with respect to Val at position 108. Conversely, the mutant I108S in the gAT_{1A} receptor actually produced a significant loss of function compared with the wild type gAT_{1A} receptor (Table 6; Fig. 8). The rank order for loss-of-function mutations in position 108 was: hAT_1 < gAT_{1A}/I108V < hAT_1/V108I < gAT_{1A} I108A < gAT_{1A} < gAT_{1A} I108S (Table 6).

We propose, based on our studies and those in the literature, an overall picture of the presumed binding pocket for the biphenylimidazole antagonists of the AT_1 receptor formulated as a two-dimensional representation (Fig. 9) or, based on the physicochemical properties of amino acid sequences, such as hydrophobicity and charges, as a helical wheel diagram (Fig. 10). When we consider the presumed three-dimensional structure of the protein, important residues widely separated in the primary sequence of the receptor appear in proximity. Most positions studied here would be predicted to face the interior of the transmembrane bundle (Fig. 10).

Discussion

We report the cloning and characterization of a gerbil Ang II receptor, highly homologous to the hAT_1 and gAT_{1A} receptors, discreetly localized to the zona glomerulosa of the adrenal gland (present results and Moriuchi et al., 1998), with similar affinity to Ang II compared with the hAT_1 receptor and with low affinity for the nonpeptidic antagonist losartan, intermediate between the hAT_1 and the gAT_{1A} receptors (Moriuchi et al., 1998). Because of these characteristics, we considered the newly cloned receptor as a gAT_{1B} receptor.

We conclude based on our gain- and loss-of-function studies of the gAT_{1A}, gAT_{1B}, and hAT_1 receptors that: 1) the most important amino acid was Val^{108}, followed by Gly^{107}, and their role was cumulative; 2) an additional very important amino acid was Leu^{195}, but only in combination with Val^{108} or Gly^{107}; and 3) we found additional but much lesser roles for Leu^{83}, Ile^{150}, Ile^{151}, and Ile^{177}.

For losartan binding to AT_1 receptors, the most important amino acids are located in TM III. We found that Gly^{107}, previously considered not to be a major contributor to losartan binding (Ji et al., 1994), is important to losartan affinity for both the gerbil and hAT_1 receptors. The S107A mutation of rAT_{1A} did not affect losartan-binding affinity (Monnot et al., 1996). It is possible that only Gly^{107} replacement has an effect on losartan binding, even though both Ala (R = CH3) and Gly (R = H) are nonpolar amino acids. This being the case, the presence of one additional carbon in the side chain in the R group could be critical for losartan binding, as is the case when Thr (2C) replacing Ser^{109} (1C) reduces the binding affinity of rAT_1 receptor for losartan by 190-fold (Ji et al., 1995).

Using gain- and loss-of-function mutations, we confirm here that Val^{108} is the most important amino acid for losartan binding to AT_1 receptors, both in gerbils and humans (Ji et al., 1995; Nirula et al., 1996; and present report). The gAT_{1A} receptor expresses both the I108V and the G107 mutations, and these are additive. On the other hand, Val^{108} is conserved in the gAT_{1B} receptor. This explains the very significant loss of function of the gAT_{1A}, and the intermediate decrease in losartan affinity of the gAT_{1B} compared with the hAT_1 receptor.

Losartan binding was severely reduced when we replaced Val^{108} with Ser, a polar amino acid, suggesting the need for a hydrophobic residue at position 108. Val^{108} may be near a general nonpeptide binding site on the AT_1 receptor, providing a hydrophobic interaction that stabilizes the nonpeptidic ligands. However, the size of the hydrophobic group seems to be important; replacing Val^{108} with an amino acid with larger hydrophobic R groups (Ile) reduced losartan binding more than a mutation with an amino acid with a smaller hydrophobic R group (Ala). Losartan may be unable to take full advantage of the hydrophobic interaction of Ile because of steric hindrance; there are four methyl groups in Ile compared with three in Val. Thus, it seems that the binding site of the nonpeptide antagonists requires nonpolar residues in the TM domains (Schambye et al., 1994). The observation that the binding characteristics of the S108V mutant of the gAT_{1A} receptor and those of the mutant S109Thr of the rAT_{1A} receptor (Ji et al., 1995) are similar supports this observation.

TABLE 5

Loss-of-function mutations in hAT_1 receptors

Data are the mean ± S.E.M. obtained from two to four experiments (each done in triplicate) after displacement of ^{125}I-Sar^1-Ang II binding by increasing concentrations of losartan, for selected hAT_1 mutant receptors transfected to COS-7 cells. To make F_{mut} values comparable, the F_{mut} values of each mutant used the wild type human EC_{50} value as a control for comparison.

Receptor	Ang II		Losartan	
	EC_{50}	F_{mut}	EC_{50}	F_{mut}
	nM		*nM*	
Wild-type				
hAT_1	4.5 ± 0.6	1.0	15 ± 2.4	1.0
gAT_{1A}	5.4 ± 0.7	1.2	5900 ± 870	393
gAT_{1B}	3.3 ± 0.4	0.7	567 ± 54	38
hAT_1 mutations: loss-of-function				
S107G	2.3 ± 0.3	0.5	40 ± 6.4	2.7
V108I	4.9 ± 0.7	1.1	550 ± 62	45
L195M	6.6 ± 0.9	1.5	12 ± 2.5	0.8
S107G/V108I	1.0 ± 0.2	0.2	2350 ± 290	160
S107G/L195M	4.3 ± 0.5	1.0	110 ± 12	7.3
V108I/L195M	3.7 ± 0.5	0.8	1180 ± 150	79
S107G/V108I/L195M	5.1 ± 1.0	1.1	6300 ± 1340	420

F_{mut}, mutant EC_{50}/hAT_1 EC_{50}.

The role of TM III in losartan binding may not be limited to that of positions 107 and 108. Another TM III residue reported to play an important role in losartan binding is Asn^{111} (Groblewski et al., 1995; Monnot et al., 1996; Groblewski et al., 1997). These residues are in the same position, based on sequence comparison analysis, as residues [such as Glu^{113}, His^{108}, Lys^{182} (Schwartz, 1994), and Asp^{113} (Strader et al., 1989)] that play important roles in ligand interaction in other G-protein–coupled receptors, highlighting their considerable structural homology.

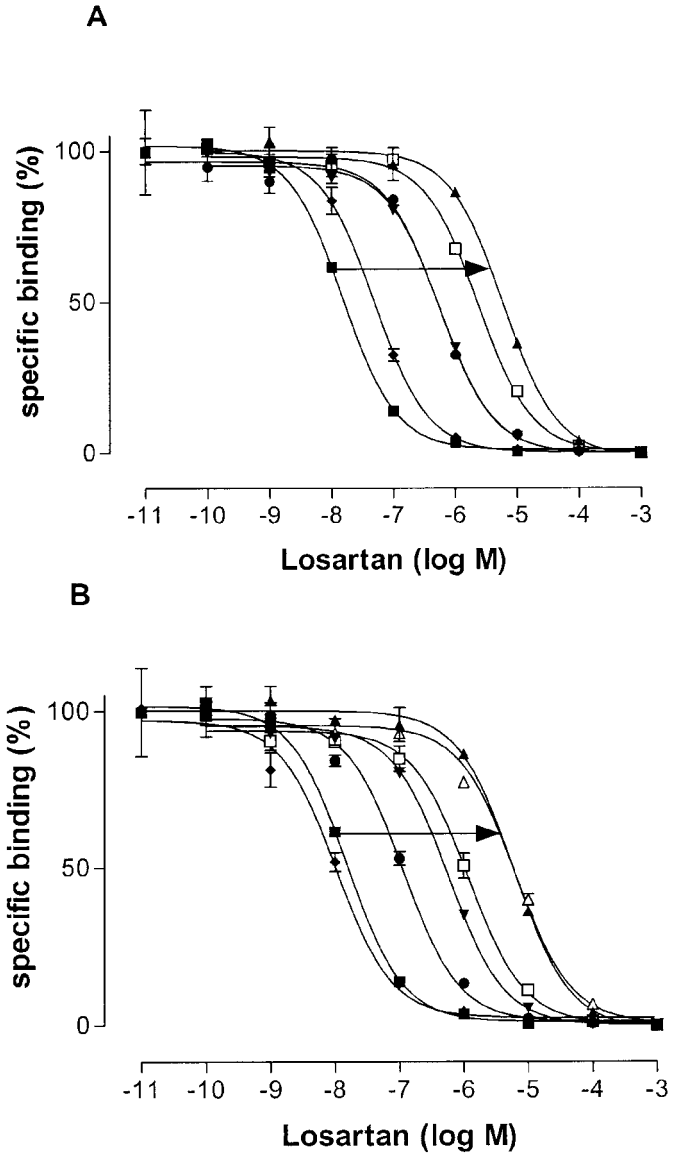

Fig. 7. A. Loss-of-function mutations in human AT_1 receptors (S107G and V108I). The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected hAT_1 mutant receptors transfected to COS-7 cells. The arrow shows the progressive loss of function of the mutated hAT_1 receptors. Single and combinatorial mutants are compared with the gAT_{1A} receptor. ■, hAT_1; ▲, gAT_{1A}; ▼, gAT_{1B}; ♦, S107G; ●, V108I; □, S107G/V108I. B, loss-of-function mutations in human AT_1 receptors (L195M and combinatorial mutations). The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected hAT_1 mutant receptors transfected to COS-7 cells. The arrow shows the progressive loss of function of the mutated hAT_1 receptors. Single and combinatorial mutants are compared with the gAT_{1B} receptor. ■, hAT_1; ▲, gAT_{1A}; ▼, gAT_{1B}; ♦, L195M; ●, S107G/L195M; □, V108I/L195M; △, V108I/S107G/L195M.

Leu^{195} in TM V is also important for losartan binding in both gerbil and human AT_1 receptors. The role of Leu^{195} is only revealed when in combination with mutations in positions 107 and 108. The effect of L195M is not only cumulative with that of S107G and/or V108I but potentiates their effect, as revealed by the introduction of the mutant V108I into the gAT_{1B} receptor, that of L195M into the AT_{1B} receptor, and by the combinatorial loss-of-function mutations in the hAT_1 receptor.

We present evidence here suggesting that the effects of positions 107, 108, and 195 might be influenced by other nonconserved amino acid residues not analyzed in the present study and that may affect the receptor conformation. For example, the gain of function of mutation G107S is different in the gAT_{1A} (10-fold) and gAT_{1B} (3-fold). The losartan affinity of the G107S/M195L double mutant gAT_{1B} receptor was still 3-fold lower than that of the hAT_1 receptor. Furthermore, the addition of the L195M mutant to the gAT_{1A} receptor decreased losartan affinity by 3.5-fold, whereas the addition of mutant V108I to the gAT_{1B} receptor reduced affinity for losartan 49-fold.

In addition to the interaction of selected amino acids in TM III and TM V, we show that mutations in TM II (V83L), TM IV (V150I and V151I), and the extracellular loop 2 (S177I) can affect losartan binding. These mutations have a relatively small effect on losartan binding when single but different and sometimes opposite effects when combined with other major mutations, such as G107S, I108V, and/or L195M.

There are reports on additional residues in other TM domains that may affect losartan binding. A natural mutation in TM IV, T163A, is probably responsible for the lower affinity for losartan in bovine, canine, ferret, and porcine receptors (Sasaki et al., 1991; Yoshida et al., 1992; Itazaki et al., 1993; Burns et al., 1994; Ji et al., 1995; Gosselin et al., 2000). In the turkey AT_1 receptor, the V163A mutation occurs in combination with mutations S107G and I108V, and this triple combination is probably responsible for the very low losartan affinity of the tAT_1 receptor, lower than that of gAT_{1A} receptor and 670-fold lower than that of rAT_{1B} (Murphy et al., 1993). These observations indicate that Ala^{163} in

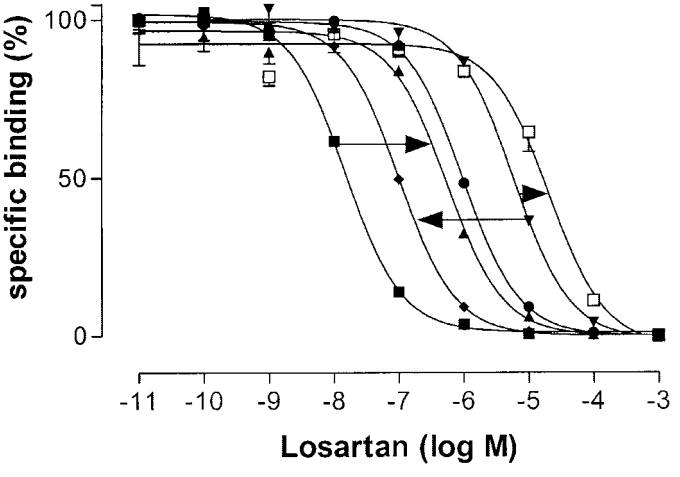

Fig. 8. Mutagenesis at position Ile^{108} in the human AT_1 and gerbil AT_{1A} receptors. The figure represents displacement of ^{125}I-Sar^1-Ang II binding, by increasing concentrations of losartan, for selected mutant receptors transfected to COS-7 cells. The arrows show the effect of the different amino acid substitutions in the hAT_1 and gAT_{1A} receptors. ■, hAT_1 Val^{108}; ▲, V108I; ▼, gAT_{1A} Ile^{108}; ♦, I108V; ●, I108A; □, I108S.

TM IV is important for losartan binding, and its effect may be enhanced by mutations in TM III. However, in both gAT_{1A} and gAT_{1B}, Ala^{163} is well conserved.

The K199A mutation in TM V decreased losartan binding (Ji et al., 1995; Monnot et al., 1996). In addition, there are reports in the literature of residues important for losartan binding in TM VI (Ser^{252}) (Ji et al., 1994, 1995; Nirula et al., 1996). According to the results from experiments of human and amphibian AT_1 chimerical receptors, the TM VII (particularly Asn^{295}) could also be an important region for losartan binding affinity without affecting Ang II binding (Schambye et al., 1994).

Our results and those of the literature, taken together, indicate that the area surrounded by TM III (Gly^{107}, Ile^{108}, Ser^{109}, Asn^{111}, and Ser^{115}), TM IV (Thr^{163}), TM V (Met^{195} and Lys^{199}), TM VI (Ser^{252}), and TM VII (Asn^{295}) could form a recession "pocket" discriminating between nonpeptidic ligands, such as losartan, and peptidic, such as Ang II. Our failure to significantly affect Ang II binding in the present experiments further supports this hypothesis. We interpret our results as suggesting that amino acids at positions 83, 107/108, 150/151, 177, and 195 are spatially proximate and they must be interacting with each other to influence losartan binding. Despite their locations on different transmembrane domains, most of these residues are positioned within a small distance of each other within the plasma membrane, suggesting that losartan binds to the mammalian AT_1 receptor in a plane that is one or two α-helical turns below the membrane surface. Such a presumed binding pocket for the biphenylimidazole AT_1 antagonists can be formulated as a two-dimensional representation (Schambye et al., 1994) or, based on such physicochemical properties of amino acid sequences as hydrophobicity and charges, as a helical wheel diagram (Murgolo et al., 1996), predicting that most positions studied here would be nearby, facing the interior of the transmembrane bundle.

The final analysis of all amino acid residues important for binding affinity of nonpeptidic antagonists is not complete. Our studies reveal some nonconserved residues that determine the molecular requirements for biphenylimidazole recognition. However, these have been reported to be not identical to nonconserved residues necessary for high-affinity binding to AT_1 antagonists from the imidazoleacrylic class (Nirula et al., 1996).

Notwithstanding, our results are noteworthy for several reasons. First, the study of a natural mutation with substantially decreased affinity for receptor antagonists provides the advantage of maintaining a close general homology with the

TABLE 6

Binding affinities of mutations in position 108 for hAT_1 and gAT_{1A} receptors

Data are the mean ± S.E.M. obtained from two to four experiments (each done in triplicate) after displacement of ^{125}I-Sar^1-Ang II binding by increasing concentrations of losartan, for selected hAT_1 mutant receptors transfected to COS-7 cells. To make F_{mut} values comparable, the F_{mut} values of each mutant used the wild type human EC_{50} value as a control for comparison.

Receptor	Ang II		Losartan	
	EC_{50}	F_{mut}	EC_{50}	F_{mut}
	nM		nM	
Wild-type hAT_1 (Val^{108})	4.5 ± 0.6	1.0	15 ± 2.4	1.0
V108I	4.9 ± 0.7	1.1	550 ± 62	45
Gerbil				
Wild-type gAT_{1A} (Ile^{108})	5.4 ± 1.2	1.2	5,900 ± 870	393
I108V	5.4 ± 0.4	1.2	101 ± 14	6.7
I108A	1.4 ± 0.3	0.3	977 ± 132	65
I108S	2.2 ± 0.4	0.5	>20,000	>1,333

F_{mut}, mutant EC_{50}/hAT_1 EC_{50}.

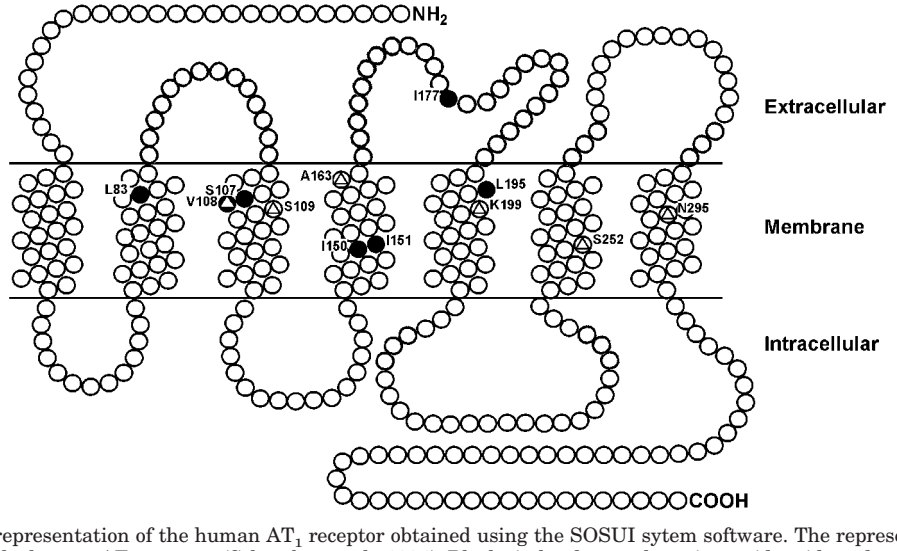

Fig. 9. Two-dimensional representation of the human AT_1 receptor obtained using the SOSUI sytem software. The representation is very similar to that described earlier for the human AT_1 receptor (Schambye et al., 1994). Black circles denote the animo acid residues described herein as important for the binding of nonpeptidic antagonists. White triangles denote residues described by other authors. A white triangle inside a black circle denotes residues described both herein and in the literature.

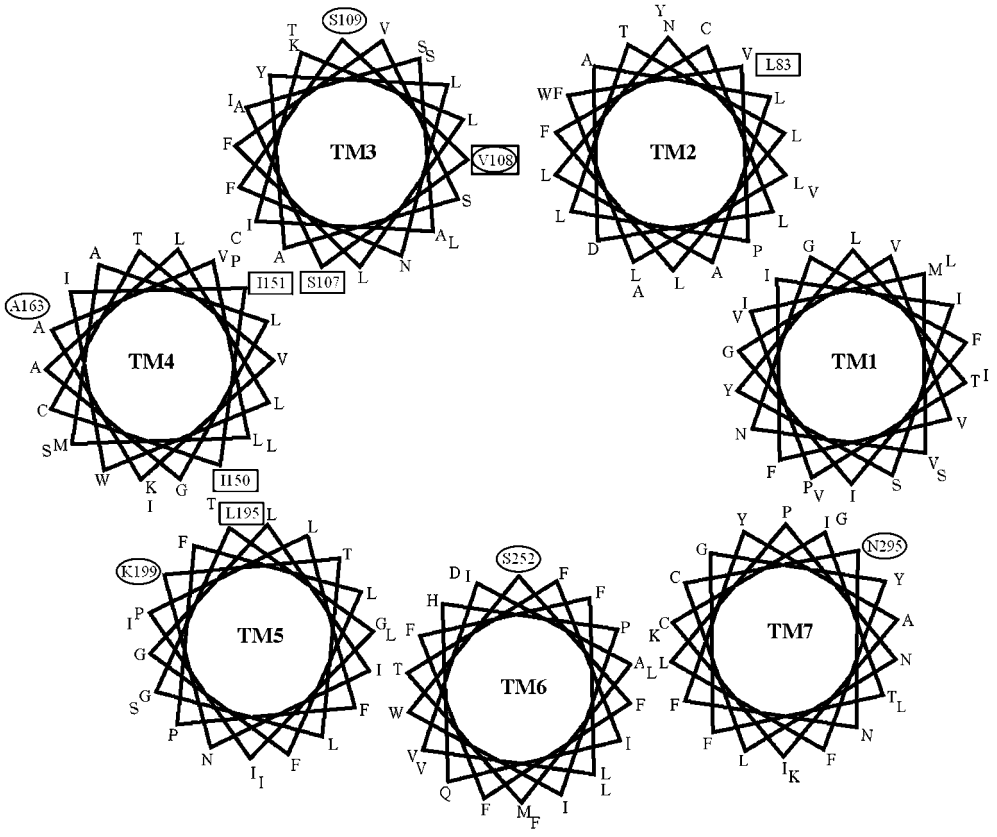

Fig. 10. Helical wheel diagram of the human AT_1 receptor drawn using the SOSUI system software. The helices were oriented with the regions of highest hydrophobicity pointing to the exterior (i.e., lipid interface) of the helix bundle and charged residues mostly facing the center of the helix bundle (Murgolo et al., 1996). Squares denote the amino acid residues described herein as important for the binding of the nonpeptidic antagonist. Circles denote residues described in the literature. A circle inside a square denotes residues described both herein and in the literature.

human AT_1 receptor with minimum three-dimensional distortion. Second, precise loss-/gain-of-function studies provided evidence that, in addition to position 108, there is a significant role for positions 107 and 195 in antagonist binding in the hAT_1 receptor.

In conclusion, we found, for both human and gerbil receptors, that the most important amino acid for losartan affinity was located in position 108 in TM III, naturally mutated in gAT_{1A} but conserved in gAT_{1B} receptors. The second most important amino acid was located in position 107 in TM III, naturally mutated in both the gAT_{1A} and gAT_{1B}. The effect of mutations in positions 107 and 108 was additive. This explains the very significant loss of function of the gAT_{1A}, and the intermediate affinity for losartan of the gAT_{1B} compared with the hAT_1 receptor. In position 108, hydrophobic residues are important, and their size may be critical for optimum losartan binding. An additional very important amino acid was located in position 195 in TM V, mutated in the gAT_{1B} but conserved in the gAT_{1A} receptor. The effect of position 195 was revealed only when the L195M mutant was combined with the G107S and/or I108V mutants, and the effects are proportionally higher when the mutants are combined. In addition, we found that, in the gerbil, some additional mutations in positions 83 in TM II, 150/151 in TM IV, and 177 in intracellular loop 2 could, when single, not affect or slightly increase losartan affinity, and, when combined with the G107S/I108V mutants, lose this property and even decrease losartan binding. Advances in the understanding of the molecular requirement for human AT_1 receptor binding to nonpeptidic antagonists could help in the development of potent and specific compounds of relevant clinical use.

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