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Loss-of-Function Polymorphic Variants of the Human Angiotensin II Type 1 Receptor

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ABSTRACT

The angiotensin II type 1 (AT_1) receptor is the primary effector for angiotensin II (Ang II), a key peptide regulator of blood pressure and fluid homeostasis. AT_1 receptors are involved in the pathogenesis of several cardiovascular diseases, including hypertension, cardiac hypertrophy, and congestive heart failure, which are characterized by significant interindividual variation in disease risk, progression, and response to pharmacotherapy. Such variation could arise from genomic polymorphisms in the AT_1 receptor. To pursue this notion, we have pharmacologically characterized seven known and putative nonsynonymous AT_1 receptor variants. Functional analysis using the cell-based assay receptor selection and amplification technology (R-SAT) revealed that three variants (AT_1-G45R, AT_1-F204S, and AT_1-C289W) displayed altered responses to Ang II and other AT_1 receptor agonists and antagonists. Agonist responses to Ang II were absent for AT_1-G45R and significantly reduced in potency for AT_1-C289W (11-fold) and AT_1-F204S (57-fold) compared with the wild-type (WT) receptor. AT_1-F204S also displayed reduced relative efficacy (57%). Quantitatively similar results were obtained in two additional functional assays, phosphatidyl inositol hydrolysis and extracellular signal-regulated kinase activation. Radioligand binding studies revealed that AT_1-G45R failed to bind Ang II, whereas cell surface staining clearly showed that it trafficked to the cell surface. AT_1-C289W and AT_1-F204S displayed reduced binding affinities of 3- and 5-fold and reduced cell surface expression of 43 and 60% of that observed for the WT receptor, respectively. These data demonstrate that polymorphic variation in the human AT_1 receptor induces loss of functional phenotypes, which may constitute the molecular basis of variability of AT_1 receptor-mediated physiological responses.

Angiotensin II type 1 (AT_1) receptors (Takayanagi et al., 1992) mediate many of the physiological effects of angiotensin II (Ang II), an endogenous octapeptide that, via the actions of the renin-angiotensin system, serves as one of the pivotal regulators of blood pressure and fluid homeostasis. The AT_1 receptor is a highly conserved seven-transmembrane (7TM) receptor expressed in most tissues, particularly in vascular and renal tissues, where receptor activation leads to vasoconstriction and water retention. The renin-angiotensin system subserves critical pathophysiological roles in several cardiovascular diseases, including hypertension, cardiac hypertrophy, congestive heart failure, diabetic nephropathy, and end-stage renal disease. AT_1 receptor antagonists, typified by the compound losartan, are efficacious in the treatment of many of these diseases (Zaman et al., 2002). These polygenic disorders are clinically characterized by significant interindividual variation in disease risk, progression, and response to AT_1 receptor-based therapeutics (Baudin, 2002).

This observed heterogeneity might arise from genomic variation within various components of the renin-angiotensin system, including the AT_1 receptor. In mice, the AT_{1a} receptor locus has been linked to blood pressure variation, and functionally distinct regulatory polymorphisms have recently been described (Wong et al., 2003). A number of studies have documented the existence of several intra- and intergenic single nucleotide polymorphisms (SNPs) in the AT_1 receptor gene in the human population (Antonellis et al., 2002; Baudin, 2002). Genetic studies have shown that some

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ABBREVIATIONS: AT_1, angiotensin II type 1; AngII, angiotensin II; TM, transmembrane; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; R-SAT, receptor selection and amplification technology; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; P-ERK, phosphorylated extracellular signal-regulated kinase; TM, transmembrane; KO, knockout.

of these SNPs are associated with human diseases, including hypertension (Stankovic et al., 2003), aortic stiffness with age in hypertensive subjects (Lajemi et al., 2001), post-translational renal dysfunction and hypertension (Abdi et al., 2001), diabetic nephropathy (Doria et al., 1997; Antonellis et al., 2002), coronary artery vasoconstriction (Amant et al., 1997), cerebrovascular disease (Losito et al., 2002), and increased left ventricular mass (Takami et al., 1998). Although these studies implicate the AT_1 receptor as a possible mediator of disease variability, they are small in size and lack replication; thus, their interpretation is limited.

Most polymorphic variations in human genes are likely to be biologically "silent" because they do not induce alterations in gene expression or function of the resulting gene products (Chakravarti, 1999). Identification of those few select variants that alter protein function is critical because such knowledge allows for hypothesis-based clinical testing. However, data regarding the pharmacology of AT_1 receptor variants are lacking. We hypothesized that one or more of the amino acid changes observed in the known nonsynonymous variants of the human AT_1 receptor might alter the biological and pharmacological properties of the receptor protein. To pursue this notion, we pharmacologically characterized these AT_1 receptor variants using radioligand binding and three different functional assays. We found that three variants (AT_1-G45R, AT_1-F204S, and AT_1-C289W) demonstrate loss of functional phenotypes. These data provide a molecular basis for functional heterogeneity of human AT_1 receptors and can guide future clinical studies designed to further define the role of the AT_1 receptor gene in human disease and pharmaco-therapeutic variability.

Materials and Methods

AT_1 Receptor Plasmids. Using PCR-based methods, the AT_1 receptor was cloned from human liver cDNA, fully sequence verified, and subcloned into the pSI expression vector (Promega, Madison, WI) for functional expression in mammalian cells. The consensus wild-type AT_1 receptor sequence was defined from GenBank accession number M93394, identical to that reported previously (Takayanagi et al., 1992). To generate the polymorphic variants, point mutations were introduced using the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA). Each variant was subcloned back into the original plasmid vector and sequenced to eliminate potential noncoding PCR-generated mutations.

Receptor Selection and Amplification Technology. R-SAT was performed as described previously (Weiner et al., 2001). Briefly, NIH/3T3 cells at 70 to 80% confluence were transfected with AT_1 receptor cDNA (25 ng of receptor and 20 ng of β-galactosidase reporter/well of a 96-well plate) using the PolyFect Reagent (QIAGEN, Valencia, CA) as described in the manufacturer's protocol. One day after transfection, ligands were added in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 2% Cyto-SF3. After 6 days, the media was aspirated off, cells were lysed, *O*-nitrophenyl-β-D-galactopyranoside was added, and the resulting absorbance was measured spectrophotometrically. All concentration response curves were performed in duplicate.

Whole-Cell Competitive Radioligand Binding Assay. Briefly, 2.5 million COS-7 cells were seeded into a p10 dish and grown in 10% fetal calf serum/DMEM overnight. After 24 h, the cells were transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol (5 μg of cDNA/p10 dish). After 1 day, the cells were seeded into 48-well plates coated with 0.25% poly-L-lysine (100,000 cells/well). Cells were washed once in ice-cold Hanks' balanced salt solution (HBSS) supplemented with 0.9 mM $CaCl_2$ and 1.05 mM $MgCl_2$ and then cooled at 4°C for 30 min. The HBSS was aspirated off and cells were incubated at 4°C for 3 h in 0.5 ml of HBSS containing the radioligand (^{125}I-Ang II 5-isoleucine at 3.33×10^{-11} M) and increasing amounts of unlabeled Ang II (each concentration point was done in triplicate). After incubation, cells were washed twice with ice-cold HBSS before the addition of 0.5 ml of lysis buffer (1.0% Triton X-100, 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, and 5 mM EDTA) for 30 min at room temperature. The lysis buffer was transferred to a vial containing 4 ml of Ultima Gold (PerkinElmer Life and Analytical Sciences, Boston, MA), vials were capped and shaken, and 1 h later total radioactivity was measured on a Tri-Carb 2900 TP liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences).

Whole-Cell Saturation Radioligand Binding Assay. Whole-cell saturation binding was performed using COS-7 cells that were transfected as described above for the competitive radioligand binding. Cells were incubated in DMEM containing various concentrations of the radioligand [tyrosyl-3,5-^{3}H]Ang II (5-L-isoleucine) at 4°C for 3 h. After incubation, cells were handled as described above for the competitive radioligand binding. Nonspecific binding was determined using 10^{-5} M Ang II.

Visualization of Cell Surface Expression of the c-Myc-Tagged AT_1 Receptor. The Myc tags were engineered into the N terminus of the various AT_1 receptors. We generated an ApaI restriction enzyme site by QuickChange mutagenesis and then ligated a DNA oligonucleotide coding for the Myc sequence with ApaI sites on both ends into this site. Except for minor differences, the assay was performed as described previously (Jensen et al., 2002). Briefly, COS-7 cells were transfected as described above for competitive radioligand binding, washed once in phosphate-buffered saline (PBS), and fixed for 7 min in PBS containing 4% paraformaldehyde at 4°C. Cells were washed once in blocking buffer (5% bovine serum albumin in PBS) and incubated for 20 min in blocking buffer before incubation in antimyc antibody (Clone 9E10; Roche Applied Science, Indianapolis, IN; 1:1000 dilution in blocking buffer). After two 5-min washes with blocking buffer, the cells were incubated for 1 h with secondary Alexa Fluor 488 goat antimouse IgG (H + L) antibody (Molecular Probes, Eugene, OR; 1:200 dilution in blocking buffer). Then the cells were washed twice in blocking buffer, once in PBS, and visualized using a Leica DM IRB fluorescence microscope (Leica, Wetzlar, Germany) (excitation filter, 450–490 nm; emission was detected using a dichromatic mirror 510) with a Leica f50 camera attached.

Total Expression of EGFP-Tagged AT_1 Receptors. The EGFP tags were engineered into the C terminus of the various AT_1 receptors. We amplified, by PCR, the coding sequence of the receptors, where the 5′ primer was flanked with a PstI restriction site and the 3′ primer was designed to remove the stop codon and insert a XhoI restriction enzyme site. The PCR product was cloned into the EGFP-N1 expression vector (BD Biosciences Clontech, Palo Alto, CA) and fully sequenced. Except for minor differences, this assay was performed as described previously (Jensen et al., 2002). Briefly, COS-7 cells were transfected as described above for competitive radioligand binding, and 48 h after transfection, the cells were detached from the plate, washed twice in PBS, and transferred to black optiplates (PerkinElmer Life and Analytical Sciences). EGFP excitation was performed at 485/10 nm, and emission was measured at 530/10 nm on a Fusion reader (PerkinElmer Life and Analytical Sciences). Background was assessed from untransfected cells, and the signals were normalized with reference to the wild-type EGFP receptor expression.

Inositol Phosphate Assay. This assay was performed as described previously (Jensen et al., 2002).

Extracellular Signal-Regulated Kinase Phosphorylation Assay. Except for minor differences, this assay was performed as described previously (Hansen et al., 2000; Theilade et al., 2002). Briefly, COS-7 cells were transfected as described above for compet-

itive radioligand binding, and on the day after transfection, they were seeded onto six-well plates. Forty-five hours after transfection, the cells were serum-starved for 3 h, incubated with agonist for 12 min at 37°C, and then lysed. SDS-polyacrylamide gel electrophoresis and immunoblotting was performed as described by Theilade et al. (2002), and the bands were visualized using the enhanced chemiluminescence system (Amersham Biosciences Inc., Piscataway, NJ). To quantify the densitometry of the bands, the gels were scanned, and the density of each band was measured using NIH Image 1.62 (http://rsb.info.nih.gov/nih-image/).

Data Analysis. All agonist data are presented as pEC_{50} values, whereas all competitive antagonist data are presented as pK_i values calculated by the formula $K_i = [(IC_{50}\ \text{observed}/1 + ([\text{agonist}]/EC_{50}\ \text{agonist})]$. All pharmacological data were analyzed using Excel (Microsoft, Redmond, WA) fit and Prism (GraphPad Software, San Diego, CA); whole-cell radioligand binding data were analyzed by one-site competitive binding analysis, and saturation-binding data, R-SAT data, phosphatidyl inositol hydrolysis, and ERK activation data were all analyzed using nonlinear regression curve fitting. Regarding statistical analyses, all values obtained for variant receptors were directly compared with those observed for the WT receptor. A Bartlett test confirmed that there were no significant differences in the variance of the compared groups of data sets. Subsequently, one-tailed, paired Student's t tests were performed. In addition, a Wilcoxon test on the same data sets resulted in the rejection of the null hypothesis, as did the t test (data not shown). All results are considered statistically significant at the $p < 0.05$ level (indicated by *).

Results

Functional Screen of AT_1 Receptor Polymorphic Variants. We identified four documented and three putative, nonsynonymous polymorphisms positioned throughout the AT_1 receptor protein (Fig. 1). The variants, including amino acid change and position, nucleotide base change and position (with nucleotide position 1 defined by the adenine of the initiating methionine in the AT_1 receptor cDNA), reported minor allele frequencies and had the following sources: documented variants, AT_1-S6P [T16C; 0.13 (Rolfs et al., 1994)], AT_1-G45R [G133A; 0.05 (Rolfs et al., 1994)], AT_1-F204S [T611C; 0.025 (Koshy et al., 2002; Anastasio et al., 2003)], and AT_1-T336M [C1007T; 0.025 (Koshy et al., 2002; Anastasio et al., 2003)]; and putative variants, AT_1-C289W [T867G; rs1064533 (http://www.ncbi.nlm.nih.gov/SNP/)], AT_1-L330M [A1006C (McCarthy et al., 2001)], and AT_1-T336P [A1006C; rs1801021 (http://www.ncbi.nlm.nih.gov/SNP/)]. We generated plasmids encoding these receptor variants by site-directed mutagenesis and determined their pharmacological properties by functional expression in the cell-based assay R-SAT. Agonist responses of the wild-type receptor to Ang II yielded robust 14 ± 6-fold responses with an average pEC_{50} of 6.9 ± 0.1 (Fig. 2 and Table 1). No response to Ang II was observed in cells transfected with the marker gene alone (data not shown). Of the seven variants tested, four displayed wild-type potencies and relative efficacies (Fig. 2 and Table 1); however, three variants displayed significantly altered agonist and antagonist responses. Regarding agonist responses, AT_1-G45R failed to illicit a functional response, AT_1-F204S displayed a 57-fold reduced potency with 57 ± 2% relative efficacy, and AT_1-C289W displayed an 11-fold lower potency yet retained full efficacy compared with the wild-type receptor (Fig. 2 and Table 1; $p < 0.05$). Regarding antagonist responses, we tested the ability of two small molecular AT_1 receptor antagonists, irbesartan and telmisartan, to inhibit Ang II-induced R-SAT responses. AT_1-F204S and AT_1-C289W showed 20 to 23- and 7 to 9-fold

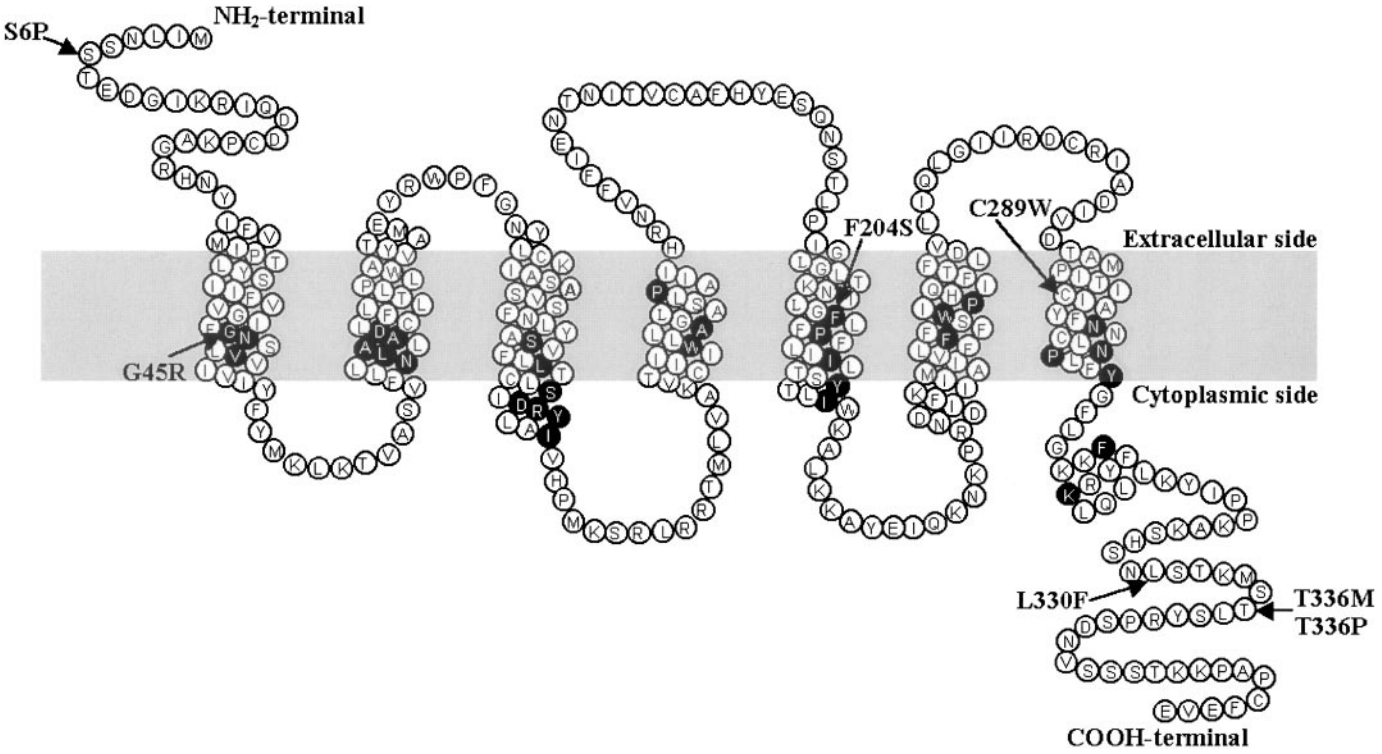

Fig. 1. AT_1 receptor variants. Black arrows indicate the amino acid locations of the identified nonsynonymous polymorphic variants of the AT_1 receptor gene. Receptor topology is predicted by alignment with the family A 7TM receptors (Baldwin et al., 1997) and visualized with reference to the crystal structure of rhodopsin (Palczewski et al., 2000). ● show the amino acids used for the receptor alignment.

reduced potencies for these drugs, respectively (Table 2; $p < 0.05$). No variants displayed increased Ang II potencies or increased basal, agonist-independent signaling (data not shown).

Agonist Pharmacology of Functionally Altered AT_1 Receptor Variants. We then tested the altered variants for their response to a panel of angiotensin peptide analogs and the angiotensin II type 2 receptor agonist CGP 42112a. The observed rank order of potency for the wild-type and variant AT_1 receptors was as follows: (Sar1, Leu8)Ang II; (Sar1)Ang II > (Val5)Ang II > Ang II > (Val 4) Ang III > CGP 42114a, which is consistent with that described previously using other functional assays (Mann et al., 1981; Sen et al., 1983).

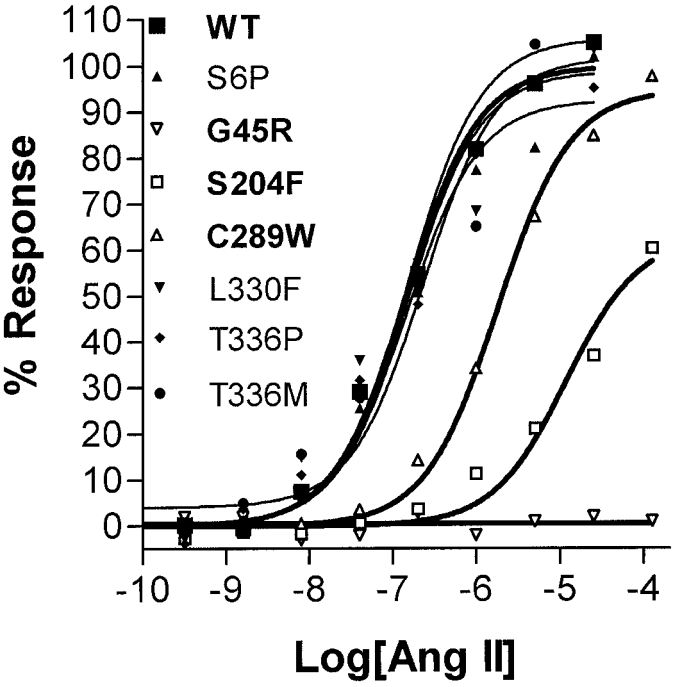

Fig. 2. Ang II response of AT_1 receptor variants. Agonist responses to Ang II for wild-type and AT_1 receptor variants were determined using R-SAT. Data shown are from representative concentration-response experiments, reported as a percentage of the maximal Ang II response observed for the WT receptor. Average pEC_{50} (± S.D.) and relative efficacies (± S.D.) values are reported in Table 1. The variant values were compared with those of the WT in a one-tailed paired Student's *t* test (*, $p < 0.05$).

Furthermore, (Sar1)Ang II, (Val5)Ang II, Ang II, and (Val 4)Ang III behaved as full agonists, whereas (Sar1, Leu8)Ang II and CGP 42114a displayed partial agonist activity (Table 2). As depicted in Fig. 3 and Table 2, AT_1-F204S and AT_1-C289W displayed significantly lower potencies than the wild-type receptor in response to all drugs tested ($p < 0.05$). AT_1-F204S consistently displayed lower efficacy ($p < 0.05$), whereas AT_1-C289W retained full efficacy for each agonist tested. AT_1-G45R did not respond to any of the compounds tested (Fig. 3 and Table 2; $p < 0.05$).

Binding and Expression Analysis of Functionally Altered AT_1 Receptor Variants. We further characterized these three functionally altered AT_1 receptor variants using radioligand binding and confocal microscopy. We performed whole-cell saturation binding using the Ang II analog [tyrosyl-3,5-^{3}H]AngII (5-L-isoleucine) to analyze the expression levels and binding affinities of the various receptors. AT_1-WT was expressed at 132 ±26 fmol/10^5cells, and AT_1-F204S and AT_1-C289W were expressed at 80 ±14 and 57 ±16 fmol/10^5cells ($p < 0.05$), respectively. AT_1-G45R did not bind (tyrosyl-3,5-^{3}H)AngII (5-L-isoleucine) (Fig. 4A). The WT receptor demonstrated high affinity for [tyrosyl-3,5-^{3}H]AngII (5-L-isoleucine) with a pK_d value of 8.1 ± 0.03, whereas AT_1-F204S and AT_1-C289W demonstrated significantly lower affinities of 7.5 ± 0.1 and 7.7 ± 0.1, respectively ($p < 0.05$) (Fig. 4B). Using whole-cell competitive binding, AT_1-F204S and AT_1-C289W displayed a pK_i value of 8.0 ± 0.1 and 8.5 ± 0.1, which represent 15- and 4-fold reductions in binding affinity compared with WT, respectively (Table 2; $p < 0.05$)

We tagged the N terminus of the various receptors with c-myc epitopes and visualized them using immunostaining with detergent-free media to ensure that only receptors expressed on the cell surface were visualized (Jensen et al., 2002). As depicted in Fig. 4C, all receptor variants were expressed at the cell surface. We then used EGFP-tagged AT_1 receptor variants to quantitate whole-cell expression levels. Several studies have shown that AT_1-GFP-tagged receptors function in a manner comparable with that of untagged AT_1 receptors (Jensen et al., 2002). A quantitative fluorometric analysis of the expression level of EGFP-tagged receptor expression in COS-7 cells showed that AT_1-WT, AT_1-F204S, and AT_1-C289W were all expressed to the same degree but that the AT_1-G45R variant was expressed at 55% of wild-type

TABLE 1

Pharmacology of AT_1 receptor variants.

Agonist studies; Ang II agonist responses were determined using R-SAT. The average pEC_{50} (± S.D.) and relative efficacy (± S.D.) values are reported. Relative efficacy was calculated as a percentage of the maximum response of the variant receptor to the Ang II response observed for the wild-type receptor in experiments performed in parallel. Competitive antagonist responses were determined using R-SAT. AT_1-F204S and AT_1-C289W receptor variants were assayed in the presence of 16 and 10 μM Ang II, respectively, and all other assays were performed with 1.25 μM Ang II. The average pK_i and S.D. for n replicate experiments are reported. Fold denotes the ratio of the potency observed for the variant to wild-type receptor. Statistical analysis utilized paired student's t-tests to determine significant differences from wild type.

	Agonist				Antagonists					
	Ang II				Telmisartan			Irbesartan		
Receptor	pEC_{50}	Fold	%Efficacy	n	pK_i	Fold	n	pK_i	Fold	n
WT	6.9 ± 0.1	1	100	9	8.6 ± 0.2	1	7	8.2 ± 0.2	1	8
S6P	6.9 ± 0.1	1	93 ± 2	5	8.6 ± 0.1	1	3	8.2 ± 0.1	1	3
G45R	N.R.*			8	N.D.			N.D.		
F204S	5.1 ± 0.2*	57	57 ± 2*	10	7.2 ± 0.2*	23	8	6.9 ± 0.2*	20	8
C289W	5.8 ± 0.1*	11	93 ± 1*	10	7.7 ± 0.2*	9	8	7.4 ± 0.2*	7	9
L330F	6.9 ± 0.1	1	106 ± 15	5	8.6 ± 0.1	1	3	8.2 ± 0.1	1	3
T336P	6.9 ± 0.1	1	99 ± 8	7	8.6 ± 0.1	1	3	8.2 ± 0.2	1	3
T336M	6.8 ± 0.1	2	102 ± 8	6	8.6 ± 0.1	1	3	8.2 ± 0.3	1	3

NR, no response; ND, not determined.
*, $P < 0.05$).

levels (Fig. 4D; $p < 0.05$). Collectively, these data demonstrate that the AT_1-G45R variant is clearly trafficked to the cell membrane, expressed at lower levels compared with the wild type, yet fails to bind Ang II. AT_1-F204S and AT_1-C289W receptors both display lower levels of cell surface expression and decreased binding affinities for Ang II analogs.

Second Messenger Analysis of the Functionally Altered AT_1 Receptor Variants. Several Ang II analogs that are capable of activating ERKs, but not Gαq proteins, and a mutant AT_1 receptor that selectively activates ERKs have been described previously (Holloway et al., 2002; Seta et al., 2002), which suggests that AT_1 receptor-mediated activation of G-proteins and ERKs can occur through independent mechanisms. We therefore analyzed these AT_1 receptor variants in two additional functional assays, ERK activation and phosphatidyl inositol hydrolysis (Gαq-dependent). AT_1-G45R did not produce any response in either assay (Fig. 5 and Table 2). In the inositol hydrolysis assay, AT_1-F204S showed a 6-fold reduction in potency with 60 ± 2% relative efficacy, whereas the AT_1-C289W displayed a 3-fold reduction in potency and full efficacy (Fig. 5A and Table 2; $p < 0.05$). Data on ERK activation were similar. To assess the potencies of ERK activation, an eight-point concentration curve was generated, and Western blot analysis of phosphorylated and total ERK was performed. Densitometric analysis of the phosphorylated ERK (P-ERK) revealed that the potency of AT_1-F204S and AT_1-C289W compared with the wild-type are 6- and 4-fold lower, respectively (Fig. 5B and Table 2; $p < 0.05$). A P-ERK densitometric analysis showed that the relative efficacy of the AT_1-F204S was 41 ± 4%, whereas AT_1-C289W showed full efficacy (Fig. 5C and Table 2; $p < 0.05$).

Discussion

In this study, we have pharmacologically characterized seven nonsynonymous polymorphic variants of the human angiotensin II type 1 receptor gene. We have characterized these receptor variants in detail and have identified three (AT_1-G45R, AT_1-F204S, and AT_1-C289W) for which the polymorphism induces a loss of a functional phenotype. These three receptor variants all display decreased affinity and functional potencies for Ang II and the antihypertensive agents ibesartan and telmisartan. Given the critical role of the AT_1 receptor in mediating human pressor responses and regulating fluid homeostasis, these data provide further in-

TABLE 2

Detailed agonist pharmacology of AT_1 receptor variants.

Pharmacological properties of the wild-type and polymorphic variant receptors are reported. *R-SAT,* data shown are average values from n concentration response experiments. Average pEC_{50} (± S.D.) and relative efficacies (± S.D.) values are reported. *IP hydrolysis,* data shown are average values from *n* concentration-response experiments. Average pEC_{50} (± S.D.) and relative efficacies (± S.D.) values are reported. *ERK activation*, relative efficacy was assessed with saturating concentrations (1 μM) of Ang II and for assessment of potency, concentration curves were performed, with densitometric gel quantification of phosphorylated ERK (pERK). *Radioligand binding assay*, binding was performed in the presence of 3.3×10^{-11}M ^{125}I-Ang II and increasing amount of unlabeled Ang II. Each concentration point was done in triplicate. K_i values were determined by one-site competitive binding analysis. The relative efficacies of the variants are calculated as a percentage of the maximal Ang II response observed for the WT receptor. Fold denotes the ratio of potency of the WT to variant receptor. Statistical analysis included one tailed paired t-test to determine significant differences from wild type.

Assay	AT_1-WT			AT_1-G45R		AT_1-F204S				AT_1-C289W			
R-SAT	pEC_{50}	%Efficacy	*n*	pEC_{50}	*n*	pEC_{50}	Fold	%Efficacy	*n*	PEC_{50}	Fold	%Efficacy	*n*
(Sar1;Leu8)Ang II	7.6 ± 0.3	26 ± 0	5	N.R.*	2	6.6 ± 0.1*	10	8 ± 1*	5	6.7 ± 0.1*	8	25 ± 2	5
(Sar1)Ang II	7.5 ± 0.2	98 ± 2	7	N.R.*	2	6.6 ± 0.2*	7	53 ± 5*	7	6.9 ± 0.1*	4	91 ± 4	7
(Val5)Ang II	7.3 ± 0.1	103 ± 7	5	N.R.*	2	5.8 ± 0.1*	36	41 ± 1*	5	6.4 ± 0.1*	8	81 ± 1*	5
(Val4) Ang III	5.3 ± 0.1	N.D.	4	N.R.*	2	4.0 ± 0.1*	21	N.D.	4	4.2 ± 0.2*	14	N.D.	4
CGP 42114a	<4.3 ± 0.3	N.D.	3	N.R.*	2	<3.9 ± 0.3	N.D.	N.D.	3	<4.1 ± 0.0	N.D.	N.D.	2
PI hydrolysis													
Ang II	9.2 ± 0.1	100	3	N.R.*	3	8.4 ± 0.1*	6	60 ± 2*	3	8.6 ± 0.1*	4	92 ± 3	3
ERK activation													
Ang II	9.6 ± 0.2	100	3	N.R.*	3	8.8 ± 0.0*	6	41 ± 4*	3	9.1 ± 0.2*	3	92 ± 2	3
Competitive binding	pKi			pKi		pKi				pKi			
Ang II	9.2 ± 0.1	N.D.	5	N.D.		8.0 ± 0.1*	15	N.D.	4	8.5 ± 0.1*	4	N.D.	5

N.R., no response; N.D., not determined.
*, $P < 0.05$).

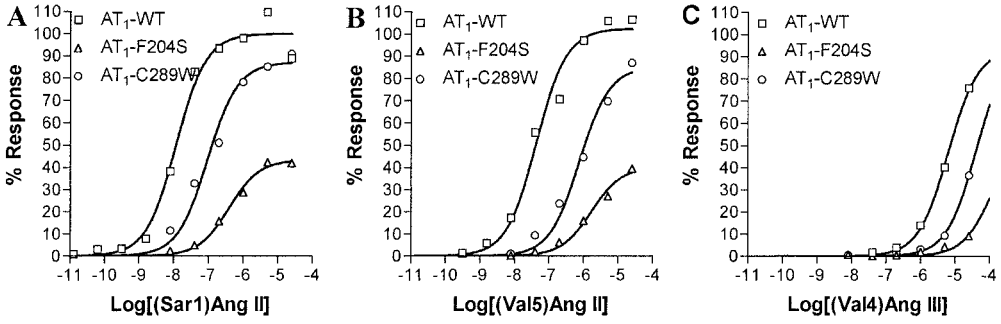

Fig. 3. Agonist pharmacology of AT_1 receptor variants. Agonist responses to Ang II and related peptides for wild-type and AT_1 receptor variants were determined using R-SAT. Data shown are from representative concentration-response experiments, reported as a percentage of the maximal Ang II response observed for the WT receptor. (Sar1)Ang II (A), (Val5)Ang II (B), and (Val4)Ang III (C) are shown for the wild-type, AT_1-F204S, and AT_1-C289W receptors. Average pEC_{50} (± S.D.) and relative efficacies (± S.D.) values are reported in Table 1. The variant values were compared with those of the WT in a one-tailed paired Student's *t* test (*, $p < 0.05$).

sights into the pharmacogenetics of human cardiovascular function and therapeutic response to commonly used AT_1 receptor antagonists.

Multiple groups have analyzed human populations for genomically encoded polymorphisms in this gene (Bonnardeaux et al., 1994; Rolfs et al., 1994; Curnow et al., 1995; Klemm et al., 1995; Antonellis et al., 2002; Koshy et al., 2002; Anastasio et al., 2003). Using PCR-based amplification and sequencing of genomic DNA from 67 persons without cardiovascular disease, Rolfs et al. (1994) identified the G-to-A transition at nucleotide position 133 responsible for the glycine-to-arginine variant at amino acid position 45. This polymorphism was present with a minor allele frequency of 0.05 in this cohort. Similar techniques were also used to detect, from a cohort of 80 subjects, the T-to-C transition that creates the phenylalanine-to-serine variant at amino acid position 204 (Koshy et al., 2002; Anastasio et al., 2003). This polymorphism was present with a minor allele frequency of 0.025 in Asians but was not detected in white or African American subjects. The observed low minor allele frequencies

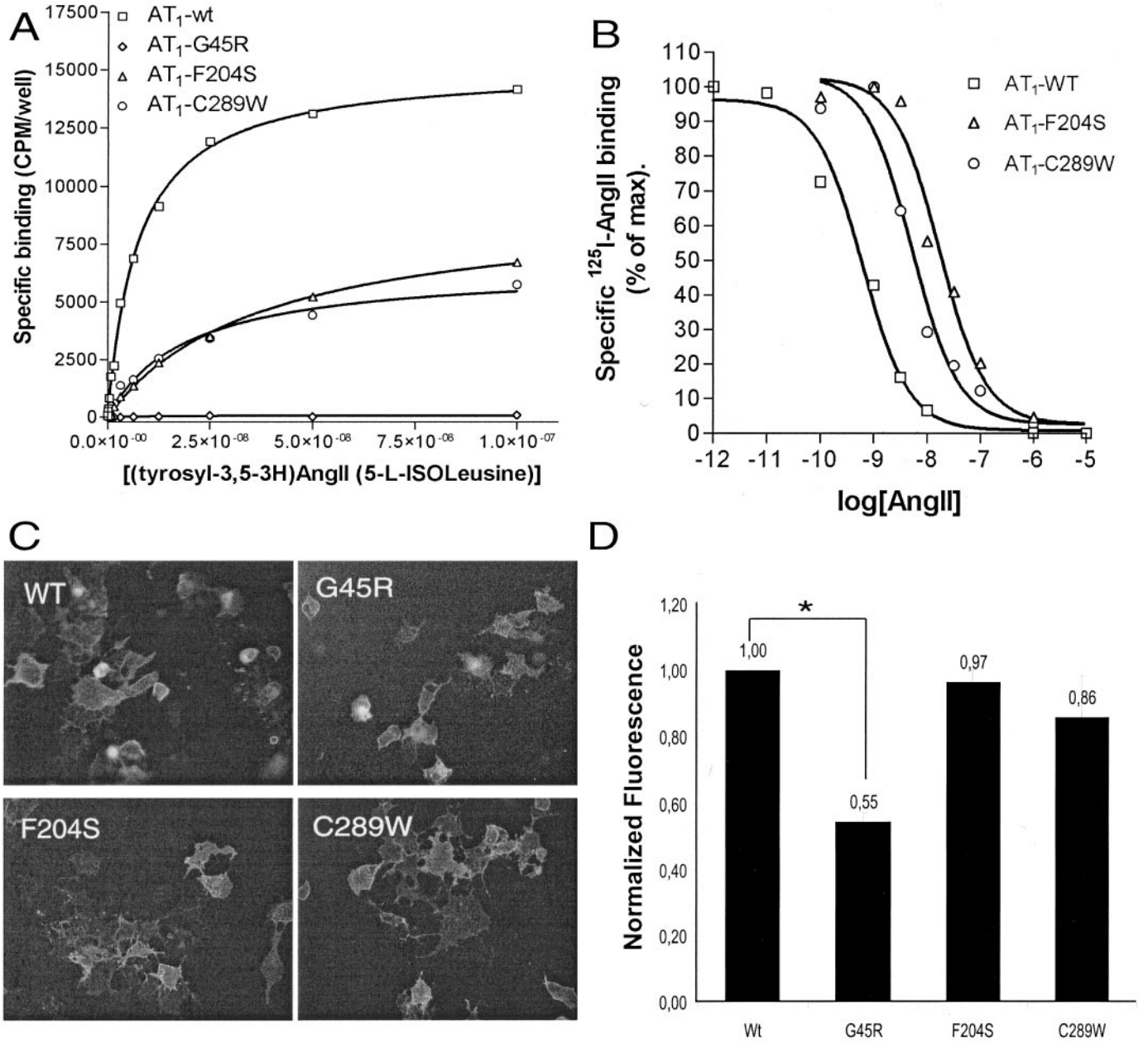

Fig. 4. Binding and cell surface expression analysis of AT_1 receptor variants. A, saturation binding was performed on the AT_1 receptor variants using [tyrosyl-3,5-^{3}H]angiotensin II (5-L-isoleucine), where nonspecific binding was determined using 10^{-5} M Ang II. Each assay was performed in triplicate; specific binding (cpm/well) versus [tyrosyl-3,5-^{3}H]angiotensin II (5-L-isoleucine)] curves that represent three separate experiments are shown. B, competitive radioligand binding assays were performed on the AT_1 receptor variants in the presence of 3.3×10^{-11} M ^{125}I-Ang II and an increasing amount of unlabeled Ang II. Each concentration point was performed in triplicate, and the specific binding of [^{125}I]Ang II versus Ang II are shown. Curves shown represent four to five separate experiments. C, visualization of cell surface expression of COS-7 cells transfected with c-myc-tagged AT_1 receptor variants, where visualized receptors appear white in the photomicrograph. D, total cellular expression of AT_1-EGFP-tagged receptor variants was determined by quantitative analysis of the total fluorescence and normalized to wild-type values (100%). Data are derived from three experiments, each performed in triplicate. Only AT_1-G45R was found to be expressed at levels statistically different than WT ($p < 0.05$)

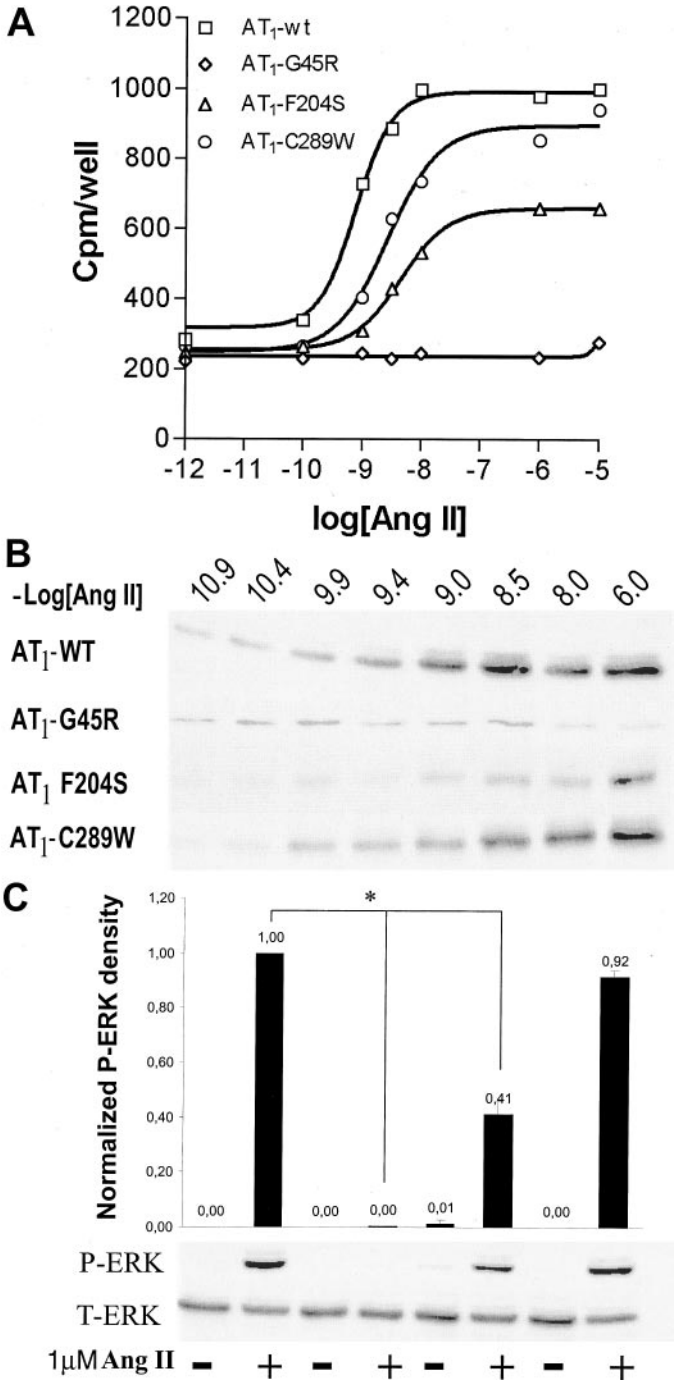

Fig. 5. Second messenger activation of the AT_1 receptor variants. A, concentration-response curves for Ang II-induced inositol phosphate accumulations are shown by representative curves from three experiments. Data are depicted (cpm/well) versus the Ang II concentration. pEC_{50} and relative efficacy values are reported in Table 2. B and C, Western blot analysis of AT_1-mediated ERK phosphorylation. Concentration-response curves of P-ERK were assessed by densitometric analysis of the P-ERK band intensity (B). pEC_{50} values, reported in Table 2, were assessed using nonlinear regression in GraphPad prism. Gels depicted represent three separate experiments. Phosphorylated and total ERK are shown for saturating doses (1 μm) of Ang II by representative images of gels from three experiments (C). To assess the relative efficacies of the variants and WT receptors, densitometric gel quantification of the P-ERK band intensities was performed, and the density of the bands was normalized with reference to the WT receptor maximum response values (100%). The values observed for the receptor variants were compared with those of the WT receptor using a one-tailed paired Student's t test (*, $p < 0.05$).

of these two variants are probably why they were not identified in separate cohorts (Bonnardeaux et al., 1994; Curnow et al., 1995; Klemm et al., 1995; Antonellis et al., 2002). The cysteine-to-tryptophan variant at amino acid position 289 was identified in GenBank (dbSNP); however, no data yet exist regarding allele frequencies for this potential polymorphic variant. Clearly, given the dramatic pharmacological phenotypes induced by these specific polymorphic variations, additional genotypic studies designed to confirm and extend these initial observations are warranted.

All three variants with altered phenotypes are located in transmembrane domains and are highly conserved, where AT_1-G45 and AT_1-F204 are conserved (>69%) across Class A 7TM receptors (Baldwin et al., 1997) and AT_1-C289 is conserved in all angiotensin receptors cloned to date (Hoe and Saavedra, 2002). The AT_1-G45R variant displays the most severe phenotype characterized by reduced but detectable cellular expression, failure to bind Ang II, and a complete lack of signaling in all three functional assays applied in this study. This is the first description of a residue in the TM1 domain of the AT_1 receptor that is critical for ligand binding. A TM1 domain nonsynonymous polymorphic variant of the endothelin B receptor with impaired binding and altered trafficking has been described and seems to be mechanistically related to Hirschsprung's Disease (Tanaka et al., 1998). Similarly, TM1 domain variants in the melanocortin 4 receptor with similar in vitro phenotypes have been associated with obesity (Farooqi et al., 2003). These observations raise the intriguing hypothesis that future studies may define G45R as a disease-related gene with regard to AT_1 receptor physiology. The phenotype observed for the AT_1-F204S variant includes reduced cell surface expression, binding affinities, and potency and efficacy in functional assays. Previous studies have implicated the extracellular-facing portion of TM5 as a region important for ligand binding. A current model proposes a direct contact between Val5 in Ang II and F204 (Nikiforovich and Marshall, 2001). F204 is near K199, a residue known to be involved in agonist and antagonist binding (Noda et al., 1995). Finally, the AT_1-C289W variant, located in the TM7 domain, displays reduced surface expression and decreases in binding affinities that are similar in magnitude to the changes in potencies observed in functional assays, suggesting that defective binding confers the observed phenotype. C289W is in a region of TM7 that has been proposed to mediate binding of the C-terminal region of the Ang II polypeptide (Perodin et al., 2002); therefore, it is not surprising that the introduction of a bulky tryptophan residue may alter receptor confirmation sufficiently enough to disturb this interaction.

The phenotypes of the human AT_1 receptor variants described herein have important pharmacogenomic implications. AT_1 receptor antagonists are widely used antihypertensive agents but therapeutic responses differ among patients (Zaman et al., 2002). The variant receptors exhibit decreased in vitro potency for these agents that is significant enough in magnitude is to possibly manifest as inadequate therapeutic responses to this class of agents in polymorphic individuals. In addition to a role in therapeutic responses to AT_1 receptor-based drugs, persons expressing these hypomorphic variants may display diminished receptor-mediated effects, consistent with a less dynamic renin/angiotensin system. The physiological manifestations of this can be inferred

from the phenotypes of mice harboring targeted deletions of the AT_1 receptor gene (Ito et al., 1995; Sugaya et al., 1995; Tsuchida et al., 1998). Compared with WT mice, heterozygotic AT_{1a} knockout (KO) mice display 10-to-12 mm Hg reductions in systolic blood pressure and transient Ang II pressor responses with preserved magnitudes. Homozygous AT_{1a} KO mice display greater reductions in blood pressure and absent Ang II pressor effects. Finally, examinations of AT_{1a}/AT_{1b} homologous double KO mice provide a different picture; for example, the mice in these examinations are characterized by decreased ex utero survival rates, low body weight, severe hypotension, and abnormal renal morphology (Tsuchida et al., 1998). If recapitulated in humans, homozygous variant carriers would be predicted to suffer from severe developmental abnormalities, whereas heterozygous expression of G45R and related variants may confer protection against hypertension.

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