Chimeric AT_1/AT_2 Receptors Reveal Functional Similarities Despite Key Amino Acid Dissimilarities in the Domains Mediating Agonist-Dependent Activation†

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ABSTRACT: Chimeric AT_1/AT_2 angiotensin II (AngII) receptors in which the sixth and/or seventh transmembrane-spanning domains of the AT_2 receptor were substituted into the AT_1 receptor were used to investigate the activation mechanisms of the two receptor subtypes. Numerous reports have identified amino acid residues in the sixth and seventh transmembrane-spanning domains of the AT_1 receptor involved in the intrareceptor activation mechanism following agonist binding. Many of these residues are not conserved in the AT_2 receptor; the corresponding AT_2 receptor residues are, in fact, disruptive of AngII-dependent activation when substituted into the AT_1 receptor. Surprisingly, the chimeric AT_1/AT_2 receptors—which also lack these crucial AT_1 residues—exhibited AngII-induced activation of phosphoinositide hydrolysis with efficacies and potencies similar to the wild-type AT_1 receptor. Consistent with earlier reports, a AT_1[Y292F] point mutant demonstrated greatly decreased agonist-induced activation of phosphoinositide hydrolysis. However, a AT_1[Y292F/N295S] double-point mutant allowed for normal agonist-induced activation with a pharmacodynamic profile indistinguishable from the wild-type receptor. Despite amino acid dissimilarities, the same corresponding domains and even the same residue loci in both of the AngII receptor subtypes are equally able to mediate agonist-induced receptor activation. This suggests that these corresponding domains in the AT_1 and the AT_2 receptors are crucial to the activation mechanism, demonstrating greater structural flexibility than previously believed regarding AT_1 receptor activation and supporting the possibility of a common activation mechanism for the two receptor subtypes.

Angiotensin II (AngII)[1] is the active component of the renin−angiotensin system, a neuroendocrine mechanism activated in times of hypovolemia, hyponatriemia, or other hypotensive conditions (*1*). Through a series of proteolytic reactions, the biologically active peptide AngII is produced, both in the periphery as well as in the brain. AngII induces its many effects on the endocrine, cardiovascular, and nervous systems by binding to and activating specific membrane-bound receptors on its target tissues (*2, 3*). Receptor activation leads, in turn, to a cascade of intracellular signaling events ultimately resulting in a number of physiological responses—vasoconstriction (*4*), increased heart rate and contractility (*5*), aldosterone release (*1*), increased thirst and fluid intake (*6*)—which attempt to restore normal blood pressure and/or electrolyte balance. Thus, AngII is extremely important in cardiovascular homeostasis, as well as one of the most potent dipsogens known (*6*).

Two pharmacologically distinct subtypes of AngII receptors have been identified (*7*) and designated type 1 (AT_1) and type 2 (AT_2). While both receptor subtypes bind the native agonist AngII with equal affinity, they do show marked differences in their abilities to bind a number of synthetic ligands. The nonpeptide antagonist losartan (*8*) selectively binds with high affinity to the AT_1 receptor, while the AT_2 receptor selectively binds both the nonpeptide antagonist PD 123319 (*9*) and the peptidic agonist CGP 42112A (*10*). Molecular cloning of both these AngII receptor subtypes (*11, 12*) has allowed for more structural comparisons. Hydrophobicity plots of the predicted amino acid sequences of the receptor subtypes revealed that they both possess a seven transmembrane-spanning domain architecture, and belong to the superfamily of heterotrimeric G protein-coupled receptors (GPCRs). Despite their ability to bind AngII with equal affinity, the AT_1 and AT_2 receptors share a low level of homology, approximately 34%, with most of the conserved amino acids scattered througout the putative transmembrane-spanning domains.

The AT_1 receptor exhibits many properties classically associated with GPCRs, including guanine nucleotide-sensitive agonist binding (*13*), desensitization following phosphorylation by G protein receptor kinases (GRKs) (*14*), and signaling via effectors commonly associated with GPCRs, including phospholipase C (*15*) and adenylate cyclase (*16, 17*). The AT_1 receptor subtype is responsible for mediating the major physiological responses attributable to AngII, and therefore a great deal of research effort has gone into elucidating the structure−function relationships of this protein. Domains of the AT_1 receptor responsible for

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[1] Abbreviations: AngII, angiotensin II; GPCR, G protein-coupled receptor; IP_3, inositol trisphosphate; SARILE, [Sar^1,Ile^8]-angiotensin II; AT_1, angiotensin II type 1; AT_2, angiotensin II type 2; TM, transmembrane-spanning domain (e.g., TM7).

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ligand binding (*18*−*20*), receptor regulation (*21*, *22*), and G protein coupling (*13*, *23*) have been identified and examined. In addition, a handful of residues proposed to be centrally involved in the intrareceptor mechanism of activation triggered by agonist binding have been extensively characterized. Specifically, reports have demonstrated the importance of Asp^{74} in the second transmembrane-spanning domain (TM2) and Tyr^{292} in the seventh transmembrane-spanning domain (TM7) in mediating the conformational changes that lead to AT_1 receptor activation (*24*, *25*). Other studies have proposed a similar importance for other residues located in other transmembrane-spanning domains: Ser^{115} in TM3 (*26*) and His^{256} and Phe^{259} in TM6 (*27*). Finally, experiments have even been performed that suggest an interaction between Asn^{111} in TM3 and Asn^{295} in TM7 which stabilizes the inactive (i.e., drug-naive) conformation of the AT_1 receptor (*28*−*30*).

Conversely, there is much less information accumulated on the structure−function relationships of the AT_2 receptor subtype, owing in part to the uncertainty regarding its physiological function, and in part to its atypical pharmacological properties. While classified as a GPCR, the AT_2 receptor does not possess guanine nucleotide-sensitive agonist binding (*12*), and its signaling pathways are still a matter of debate, with researchers having reported contradictory results (*12*, *31*, *32*). Furthermore, there is no evidence to suggest that the AT_2 receptor internalizes (*33*) or is regulated by phosphorylation. The physiological role of the AT_2 receptor is still uncertain, although it does possess antiproliferative (*34*) and pro-apoptotic activities (*35*) and has been proposed to be involved in tissue development and remodeling. Some recent reports have begun characterizing the ligand binding domains of the AT_2 receptor (*36*−*38*), as well as the domains involved in G protein interaction (*39*). However, no studies to date have addressed the structural determinants which mediate the intrareceptor mechanism of AT_2 receptor activation.

The use of chimeric AT_1/AT_2 receptors has been valuable in determining the functions of the various domains of each of the receptor subtypes. Wang et al. (*23*) used chimeric AT_1/AT_2 receptors to demonstrate that the AT_1 third intracellular loop is responsible for the ability of the AT_1 receptor to couple to its intracellular signaling pathways. Furthermore, a previous report from our group (*40*) used chimeric AT_1/AT_2 receptors to show that the amino termini of either receptor subtype are necessary and interchangeable for the ability to bind AngII, despite the great differences in amino acid sequence between them. This latter study has shown that functional similarities can exist even between receptor domains that nevertheless possess great dissimilarities in sequence. While some of the aforementioned crucial residues for AT_1 receptor activation are conserved in the AT_2 receptor, a number of them are not. In the present study, we employed a chimeric AngII receptor-based strategy to investigate both the veracity of the currently proposed mechanism for AT_1 receptor activation as well as the potential involvement of specific AT_2 domains in receptor activation. Our results have shown that, despite clear amino acid sequence differences, the same domains and even the same corresponding residue positions in both receptor subtypes can mediate activation of signaling in response to AngII. This indicates that the structural determinants required for agonist-induced AT_1 receptor activation may not be as rigid as previously thought. Furthermore, these results may suggest a common mechanism of agonist-induced receptor activation for both AngII receptor subtypes.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture medium and supplements, including LipofectAMINE reagent, were obtained from Life Technologies (Gaithersburgh, MD). Tissue culture flasks and instruments were purchased from Fisher Scientific (Pittsburgh, PA). [^{3}H]-Inositol was obtained from American Radiolabeled Chemicals (St. Louis, MO), and [^{125}I]-angiotensin II was obtained from Amersham−Pharmacia (Piscataway, NJ). Angiotensin II and all other peptide ligands were obtained from Peninsula Labs (Belmont, CA). All other chemicals were purchased from Sigma−Aldrich (St. Louis, MO) unless otherwise noted.

Cell Culture and Transfections. COS-1 cells were grown in polystyrene tissue culture flasks in medium consisting of D-MEM (high glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C. Wild-type AT_1, AT_2, and mutant receptor cDNAs were later introduced into the COS cells by transfection with LipofectAMINE. Briefly, the growth medium was removed from the COS cells upon reaching approximately 80% confluence and replaced with transfection medium (unsupplemented D-MEM containing 1.3 μg/mL of the selected cDNA and 5.5 μL/mL LipofectAMINE) for 5 h. Following the 5 h transfection interval, the transfection medium was removed and replaced with normal growth medium. Radioligand binding or IP_3 release assays were then performed 48 h following the transfection interval.

Mutagenesis. A modified version of the splicing by overlap extension (SOE) technique was used to generate the AngII receptor chimeras. This procedure involved two steps: (1) amplification of individual fragments encoding the desired portions of each receptor using specifically designed complementary and overlapping primers, followed by (2) purification and splicing of the fragments using the polymerase chain reaction (PCR). As a refinement to enhance the fidelity of SOE, a small amount of Pfu DNA polymerase (1:100 Pfu:*Taq* ratio) was added. Briefly, the two fragments were first amplified by PCR using specially designed complementary and overlapping primers that introduced the desired mutation. The two fragments were then used along with distal primers in a PCR to produce the final product. The primers used were as follows: AT_1[AT_2 CT], 5′-ACGGCTTTGTTGGAAACCGCTTCCAGC-3′ (forward sense primer) and 5′-CGGTTTCCAACAAAGCCGTAAAACAGAGGGTTC-3′ (reverse antisense primer); AT_1[AT_2 TM7-CT], 5′-CGTGGACACTGCACTTCCTTTTGCCATCC-3′ (forward sense primer) and 5′-GGAAGTGCAGTGTCCACGATGTCG-3′ (reverse antisense primer); AT_1[AT_2 TM7], 5′-GTATTGTTTTCTCGGGAAAAAATTTAAAAAG-3′ (forward sense primer) and 5′-TTTTTTCCCGAGAAAACAATACAGGAAGGGATTAA-3′ (reverse antisense primer); AT_1[AT_2 TM6-CT], 5′-ATCTTCAGGATGGCAGCTGCTGTTGTGTTG-3′ (forward sense primer) and 5′-GCAGCTGCCATCCTGAAGATGTCATCATTTCTT-3′ (reverse antisense primer); AT_1-[Y292F], 5′-AGCGTTTTTTAACAACTGCCTGAACCC-3′

(forward sense primer) and 5′-GGCAGTTGTTAAAAAACGCTATGCAGATGGTTATGGG-3′ (reverse antisense primer); AT_1[N295S], 5′-CGTATTTTAACAGCTGCCTGAACCCTCTGTTTT-3′ (forward sense primer) and 5′-CAGGCAGCTGTTAAAATACGCTATGCAGA-3′ (reverse antisense primer); AT_1[Y292F/N295S], 5′-GCGTTTTTTAACAGCTGCCTGAACCCTCTGTTTT-3′ (forward sense primer) and 5′-CAGGCAGCTGTTAAAAAACGCTATGCAGATGGTTATGGG-3′ (reverse antisense primer). The first fragment was generated using primers T7 and a reverse antisense primer, while the second fragment was produced using primers SP6 and a forward sense primer. Wild-type AT_1 and AT_2 cDNA served as the template in these PCRs for all the mutants generated except for AT_1[AT_2 TM7], which used AT_1[AT_2 TM7-CT] as a template. Reaction conditions were 30 cycles of 94 °C (1 min), 55 °C (1 min), and 72 °C (1 min). Following purification using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI), the two fragments were combined in the overlap extension reaction using the same PCR conditions as described. Following production of the full-length chimeric receptor using SOE, the chimera was subcloned into the expression vector pCR3 (Invitrogen, Carlsbad, CA) and sequenced to confirm its validity.

Inositol Trisphosphate Assay. Transfected COS cells were loaded with [^{3}H]-inositol (4.5 μCi/mL D-MEM) for 18 h prior to assay. Transfected cells were then stimulated with agonist for 30 s, rinsed once with ice-cold phosphate-buffered saline, and then rapidly lysed in 1 mL of 10% trichloroacetic acid. Insoluble materials were pelleted at 16000*g*. The pellets were solubilized in 500 μL of 1% sodium dodecyl sulfate in 0.1 M NaOH for protein quantification. The supernatant from each lysate was extracted 5 times with 2 volumes of water-saturated ether. Following the final extraction, the aqueous layers were neutralized by addition of sodium bicarbonate and EDTA to final concentrations of 6 and 15 mM, respectively. The aqueous supernatants were added to 1 mL AG 1-X8 anion-exchange resin columns (Bio Rad Labs, Hercules, CA), and inositol phosphates were separated by stepwise elution with increasing concentrations (0−1 M) of ammonium formate in 0.1 M formic acid (*41*). The amount of IP_3 eluted from each column was quantitated by liquid scintillation counting. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Radioligand Binding Assay. Transfected COS cells were harvested by scraping into PBS and pelleting the cells by centrifugation at 23000*g* for 10 min. The cells were then resuspended in assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.3 TIU/mL aprotinin, and 100 μg/mL 1,10-phenanthroline) and lysed by polytron homogenization. Following a second centrifugation at 40000*g* for 20 min to pellet the cell membranes, the final membrane pellet was resuspended in assay buffer, and protein content was determined spectrophotometrically. The binding assays were initiated by addition of the desired amount of membrane protein (5−10 μg for the wild-type AT_1 and AT_2; 50−250 μg for the mutant AngII receptors) to assay mixture containing various concentrations of [^{125}I]-AngII and unlabeled competitors. Nonspecific binding was defined as the amount of radioligand binding remaining in the presence of 1 μM SARILE. The binding assays proceeded for 60 min and were terminated by rapid filtration using a Brandell harvestor. Radioligand binding was quantitated by gamma counting of the filters. Data were analyzed and fit to a single-site model using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Expression and AngII Binding Properties of the Chimeric AT_1/AT_2 Receptors. The chimeric AT_1/AT_2 receptors were constructed as shown in Figure 1. The cytoplasmic tail of the AT_1 receptor was replaced with that of the AT_2 receptor to make AT_1[AT_2 CT]; then adjacent AT_1 domains were progressively replaced with the corresponding AT_2 domains in the subsequent chimeras (as indicated in the nomenclature used to describe them). The cDNAs encoding the wild-type AT_1, AT_2, and chimeric receptors were each transiently transfected into COS cells, and their affinities for AngII and levels of cell surface expression were quantitated by saturation radioligand binding with [^{125}I]-AngII. The calculated K_D and B_{max} values for each of the receptors are listed in Table 1. Both of the wild-type receptors possessed similar affinities for AngII, as well as similar levels of expression. In comparison to the wild-type receptors, the AT_1/AT_2 chimeras varied in their saturation binding parameters and generally showed somewhat reduced AngII affinities and expression levels—especially those chimeras that contain substitutions of the transmembrane-spanning domains. Of all the chimeric receptors, AT_1[AT_2 CT] bound AngII the most like the wild-type receptors, with a similar K_D (3.3 ± 0.3 nM) and a slightly reduced B_{max} (2.5 ± 0.1 pmol/mg of protein) compared to the wild-type receptors. The other chimeras showed between 3-fold and 6-fold decreases in AngII affinity relative to the AT_1 receptor, as well as decreased levels of expression—with the exception of AT_1[AT_2 TM7-CT], which expressed at a slightly higher level than the AT_1 receptor. Despite the variation observed among the K_D and B_{max} values for the chimeras, the saturation binding results clearly show that they were all expressed and inserted at the cell surface such that they could recognize and bind the native ligand with relatively high affinity.

AngII-Dependent Activation of the Chimeric AT_1/AT_2 Receptors. The receptors were then tested for their ability to activate and stimulate phosphoinositide hydrolysis in transfected cells treated with a short (30 s) pulse of 1 μM AngII (Figure 2). The wild-type AT_1 receptor produced a robust increase in IP_3 release (379 ± 27.0% of basal IP_3 level) when treated with the agonist. Cells transfected with the AT_2 receptor showed no changes in IP_3 levels upon treatment with AngII—not surprising since the AT_2 receptor does not signal through stimulation of phospholipase C. Substitution of the AT_1 receptor cytoplasmic tail with that of the AT_2 receptor to make AT_1[AT_2 CT] had little discernible impact on the ability of that chimera to activate, as the maximum increase in IP_3 release mediated by AT_1-[AT_2 CT] (393 ± 25.7% of basal IP_3 level) was similar to that of the AT_1 receptor. Further substitution of both the AT_1 receptor cytoplasmic tail and the seventh transmembrane-spanning domain (TM7) with the corresponding regions of the AT_2 receptor also resulted in a chimeric AngII receptor—AT_1[AT_2 TM7-CT]—which was able to strongly activate IP_3 release (538 ± 51.6% of basal IP_3 level) in response to AngII. The fact that AT_1[AT_2 TM7-CT] activated in an AngII-dependent manner is somewhat surprising: AT_1[AT_2 TM7-

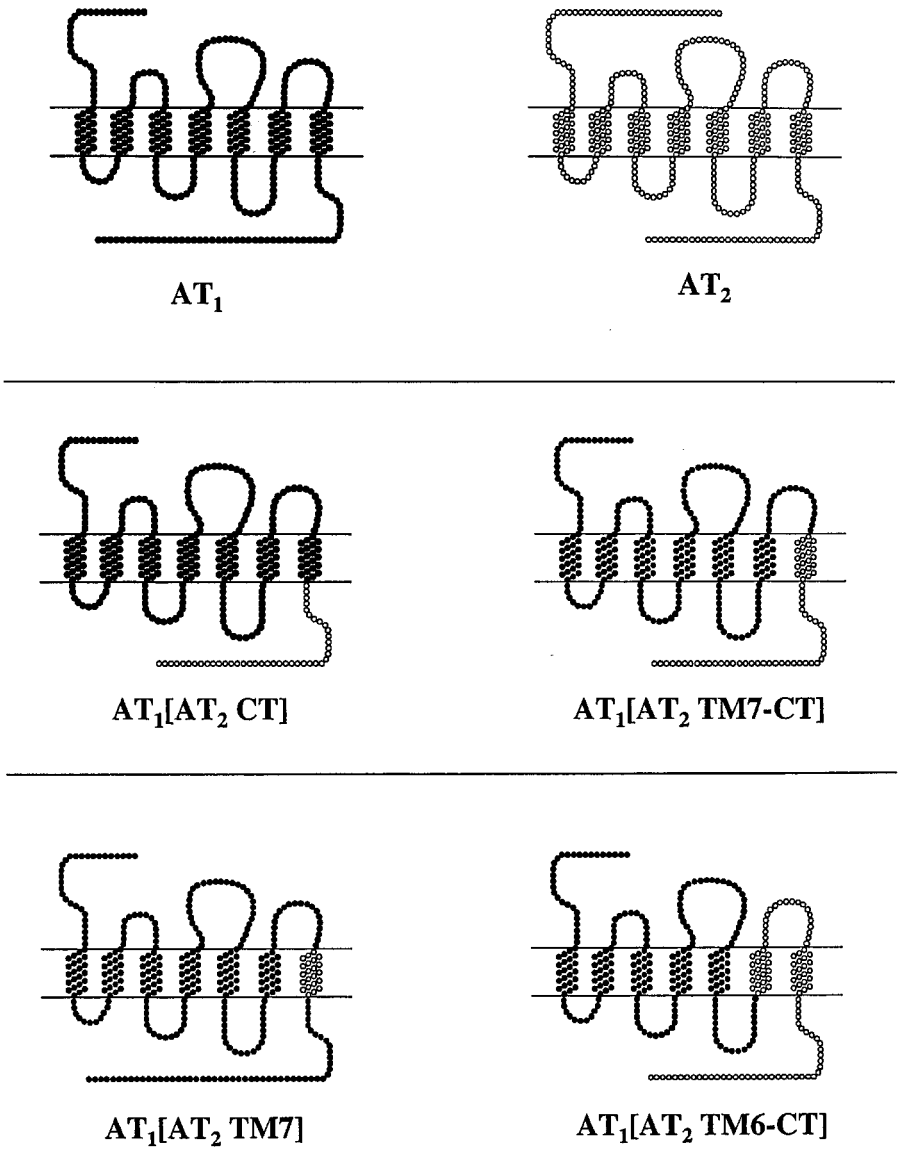

FIGURE 1: Schematic of wild-type AT_1 and AT_2 receptors and the chimeric AT_1/AT_2 receptors. Mutant receptors were constructed by a modified version of the splicing by overlap extension technique as described under Experimental Procedures.

Table 1: Saturation Binding of [^{125}I]-AngII to Wild-Type AT_1 and AT_2 Receptors and Chimeric AT_1/AT_2 Receptors Expressed in Transfected COS Cells[a]

receptor	K_D (nM)	B_{max} (pmol/mg of protein)
AT_1	5.3 ± 1.4	7.0 ± 0.9
AT_2	4.7 ± 1.8	4.1 ± 0.6
$AT_1[AT_2$ CT]	3.3 ± 0.3	2.5 ± 0.1
$AT_1[AT_2$ TM7-CT]	28.0 ± 7.8	10.9 ± 3.1
$AT_1[AT_2$ TM7]	25.3 ± 9.8	1.0 ± 0.3
$AT_1[AT_2$ TM6-CT]	16.6 ± 3.2	0.40 ± 0.02

[a] Receptor binding data were fit to a single-site model by nonlinear regression analysis. Values reported are the mean ± standard error of at least 3 independent experiments performed in triplicate.

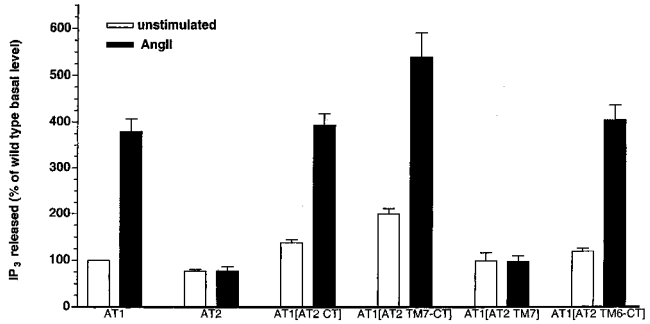

FIGURE 2: Efficacy of wild-type and chimeric receptors to activate release of IP_3 in transfected COS cells. Cells were metabolically labeled with [^{3}H]-inositol as described under Experimental Procedures and then treated with 1 μM AngII for 30 s. The values reported represent the mean ± standard deviation of 4−11 independent experiments.

CT] lacks two key amino acid residues located in TM7 of the AT_1 receptor that were previously shown to be important for AngII-dependent activation (*25, 28*). Because these residues, Tyr^{292} and Asn^{295}, are not conserved in the AT_2 receptor, they are absent from $AT_1[AT_2$ TM7-CT] as well. The result of the functional assay of $AT_1[AT_2$ TM7-CT] demonstrates that TM7 of the AT_2 receptor can, nevertheless, adequately participate in the intrareceptor mechanism of activation. The greater apparent efficacy of $AT_1[AT_2$ TM7-CT] to activate IP_3 release (Figure 2) is exagerrated in part by an elevated basal activation (199 ± 12.5%)—nearly double that of the wild-type AT_1 receptor.

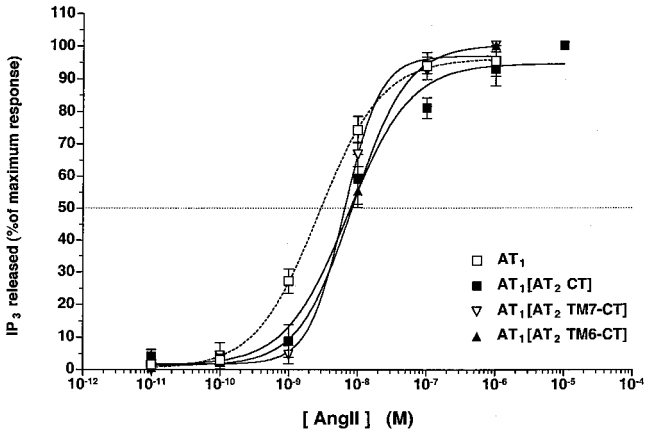

FIGURE 3: Dose−responses of wild-type AT_1 and chimeric receptors to activate release of IP_3 in transfected COS cells. Cells were metabolically labeled with [^{3}H]-inositol and treated with increasing concentrations of AngII for 30 s. The values reported represent the mean ± standard error of 3−5 independent experiments.

Curiously, when only TM7 of the AT_1 receptor was replaced with the corresponding AT_2 receptor domain yet the AT_1 cytoplasmic tail was left unaltered, the resultant chimera (AT_1[AT_2 TM7]) was no longer able to stimulate IP_3 release. It would appear either that some portion of the AT_2 receptor cytoplasmic tail must also be present for TM7 of the AT_2 receptor to properly participate in the intrareceptor activation mechanism, or simply that the novel structural junction of the AT_1 receptor cytoplasmic tail with TM7 of the AT_2 receptor may have introduced a conformational twist that uncouples AT_1[AT_2 TM7] from G_q. In radioligand binding assay, 100 μM GppNHp reduced specific [^{125}I]-AngII binding to AT_1[AT_2 TM7] by 14.8 ± 1.8%, compared to 33.8 ± 3.3% reduction in [^{125}I]-AngII binding to the wild-type AT_1 receptor. Student−Newman−Keuls post-hoc analysis of ANOVA confirms the reductions to be significant in both cases ($P < 0.01$). The fact that GTP analogues can still reduce AngII binding to AT_1[AT_2 TM7] indicates that it is still at least partially coupled to G proteins; thus, any uncoupling caused by the mutation in AT_1[AT_2 TM7] may be restricted to G_q.

Finally, even further exchange of TM6 through the cytoplasmic tail of the AT_1 receptor with the corresponding domains of the AT_2 receptor, as seen in AT_1[AT_2 TM6-CT], still resulted in a receptor that was able to activate signaling in a manner similar to the wild-type AT_1 receptor (Figure 2). AT_1[AT_2 TM6-CT] displayed both a basal level of activation (119 ± 6.2% of basal IP_3 level) similar to the AT_1 receptor, as well as an AngII-induced increase of the same magnitude (403 ± 31.8% of basal IP_3 level). As in the case for TM7 of the AT_2 receptor, TM6 of the AT_2 receptor contains some structural element(s) that compensate(s) for the absence of key AT_1 activation residues (His^{256} and Phe^{259}) (*42*) located in the corresponding AT_1 receptor domain, thereby preserving the normal AngII-dependent receptor activation mechanism.

A more detailed dose−response analysis of AngII-induced receptor activation for the AT_1 receptor and the chimeras (Figure 3) revealed only small (3−4-fold) shifts in EC_{50} values from the wild-type AT_1 receptor to the chimeras: the AT_1 receptor displayed an $EC_{50} = 2.4 \pm 0.4$ nM, while AT_1-[AT_2 CT], AT_1[AT_2 TM7-CT], and AT_1[AT_2 TM6-CT] had $EC_{50} = 8.8 \pm 2.7$, 7.12 ± 0.1, and 9.65 ± 1.8 nM, respectively. This further supports that AT_2 receptor domains—specifically TM6 and TM7—which are different in amino acid sequence from their corresponding AT_1 receptor domains can, nevertheless, be a functional equivalent with respect to the agonist-induced activation mechanism.

Table 2: Saturation Binding of [^{125}I]-AngII to Point Mutant AT_1 Receptors Expressed in Transfected COS Cells[a]

receptor	K_D (nM)	B_{max} (pmol/mg of protein)
AT_1[Y292F]	3.3 ± 0.3	4.9 ± 0.1
AT_1[N295S]	9.6 ± 1.6	8.1 ± 2.6
AT_1[Y292F/N295S]	7.2 ± 0.9	22.2 ± 1.7

[a] Receptor binding data were fit to a single-site model by nonlinear regression analysis. Values reported are the mean ± standard error of at least 3 independent experiments performed in triplicate.

Functional Consequences of Individual and Combined Point Mutations of Key Residues in the AT_1 Receptor. The functional results obtained with the chimeras are especially surprising when considered in the context of earlier mutagenesis studies done on AT_1 receptor activation. The previous studies which revealed the importance of Tyr^{292} and Asn^{295} in the AngII-dependent activation of the AT_1 receptor did so by selectively mutating these residues into the corresponding residues in the AT_2 receptor (Phe^{308} and Ser^{311}, respectively). Either mutation disrupted the normal, AngII-dependent activation of the AT_1 receptor (*25*, *28*). The natural conclusion would therefore be that the selected AT_2 receptor residues were simply insufficient to participate in and preserve the normal mechanism of AT_1 receptor activation. However, as the results of AT_1[AT_2 TM7-CT] and AT_1[AT_2 TM6-CT] demonstrate, those AT_2 receptor residues are well able to allow for agonist-dependent receptor activation. It is important to note that the study presented here differs from those previously done in that our chimeric receptors, by virtue of their construction, have resulted in substitutions at both loci (Tyr^{292} to Phe and Asn^{295} to Ser) simultaneously. To more clearly determine how making simultaneous point mutations of Tyr^{292} and Asn^{295} would affect the ability of the AT_1 receptor to activate in response to AngII, a trio of AT_1 receptor point mutants were constructed—both the individual AT_1[Y292F] and AT_1[N295S] point mutants and then a combined double-point mutant, AT_1[Y292F/N295S]. Saturation radioligand binding done on these receptors (Table 2) revealed them to possess similar affinities for AngII as the wild-type AT_1 receptor (Table 1), and levels of expression that were similar (AT_1[Y292F]) if not greater (AT_1[N295S] and AT_1[Y292F/N295S]) than that of the AT_1 receptor.

These AT_1 receptor point mutants were then tested for their ability to undergo activation and stimulation of IP_3 release (Figure 4) upon treatment with AngII. Consistent with the previous report (*25*), mutation of Tyr^{292} to Phe resulted in a receptor that produced comparatively little IP_3 release (145 ± 8.0% of basal IP_3 level) at saturating concentrations of AngII when compared to the wild-type AT_1 receptor (379 ± 27.0% of basal IP_3 level). As the AngII binding properties of the AT_1[Y292F] mutant were not impaired (Table 2), the result of the functional assay supports the long-standingnotion that Tyr^{292} plays an important role in AT_1 receptor activation. While we observed no apparent constitutive activation of IP_3 release in the AT_1[N295S] mutation, we did observe a moderately blunted maximum IP_3 response to AngII (261

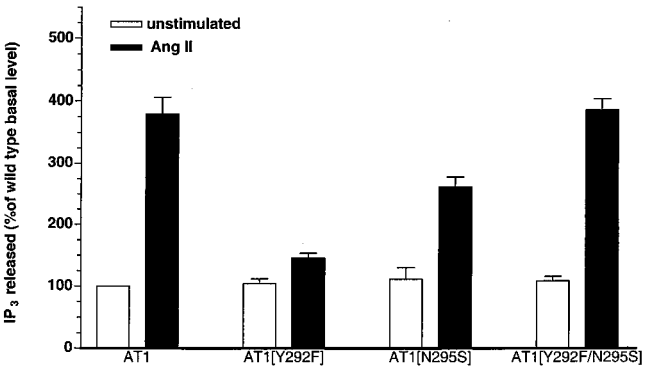

FIGURE 4: Efficacy of point mutant AT_1 receptors to activate release of IP_3 in transfected COS cells. Cells were metabolically labeled with [^{3}H]-inositol as described under Experimental Procedures and then treated with 1 μM AngII for 30 s. The values reported represent the mean ± standard error of 4−5 independent experiments.

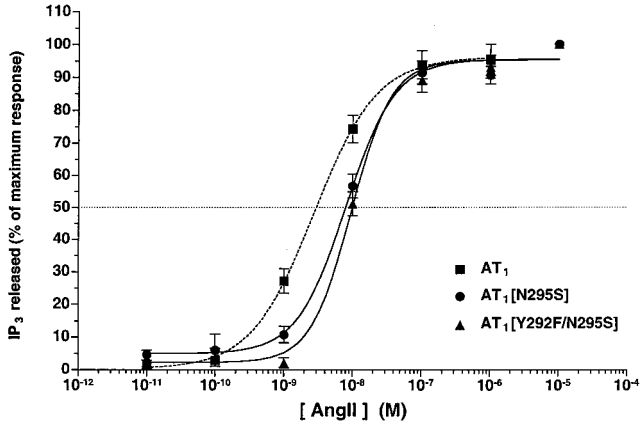

FIGURE 5: Dose−responses of wild-type and point mutant AT_1 receptors to activate release of IP_3 in transfected COS cells. Cells were metabolically labeled with [^{3}H]-inositol and treated with increasing concentrations of AngII for 30 s. The values reported represent the mean ± standard error of 3−5 independent experiments.

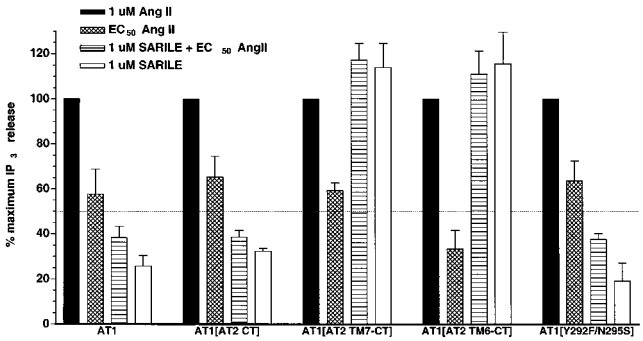

FIGURE 6: Enhanced agonist activity of SARILE at transmembrane-substituted chimeras. The ability of 1 μM SARILE to block (striped bars) and/or mimic (white bars) the effects of AngII at the AT_1 and the mutant receptors was determined and compared to the respective maximal (at 1 μM AngII, black bars) and half-maximal (at EC_{50} concentration of AngII, cross-hatched bars) IP_3 responses. The results shown represent the mean ± standard error of 3 independent experiments.

± 15.9% of basal IP_3 level) which was consistent with the results reported by Balmforth et al. (*28*). The basis for the blunted activation seen with the AT_1[N295S] mutant is unknown. It is unlikely, however, to be the result of the minor changes observed in AngII binding properties (Table 2), which indicate wild-type AngII affinity and increased expression for the AT_1[N295S] mutant. Curiously, when the "blunting" Asn^{295} mutation was made concurrent with the inactivating Tyr^{292} mutation, as in the AT_1[Y292F/N295S] double-point mutant, the ability of the receptor to activate in response to AngII was rescued—1 μM AngII produced a similar increase in IP_3 release through AT_1[Y292F/N295S] (385 ± 16.8% of basal IP_3 level) as it does through the wild-type AT_1 receptor. While this result is surprising, it is largely consistent with the results obtained with our chimeric AT_1/AT_2 receptors. It would appear that whatever conformational aberration introduced by mutation of Tyr^{292} to its corresponding AT_2 receptor residue is compensated for or corrected by the concurrent mutation of Asn^{295} to its corresponding AT_2 receptor residue. This is evidenced in the results both with the double-point mutant (Figure 4) as well as with the AT_1/AT_2 chimeras (Figure 2). A more careful dose−response examination of AngII-induced IP_3 formation, again, shows only minor shifts (Figure 5) in the AT_1[N295S] and AT_1[Y292F/N295S] EC_{50} values relative to those of the wild-type AT_1 receptor: AT_1[N295S] had an EC_{50} = 8.1 ± 2.5 nM, and AT_1[Y292F/N295S] had an EC_{50} = 10.0 ± 1.2 nM.

Pharmacodynamics of SARILE at Wild-Type and Mutant Receptors. The AngII analogue [Sar^1,Ile^8]-angiotensin II (SARILE) behaves primarily as an antagonist at the wild-type AT_1 receptor and blocks AngII-induced activation; at high concentrations, however, SARILE has been found to possess its own weak agonist activity. An earlier report on the AT_1 receptor (*29*) documented how mutation of residues involved in its activation can dramatically alter the pharmacodynamic effect of AngII receptor antagonists such as SARILE. More specificially, mutation of Asn^{111} was found to substantially increase the weak agonist activity of SARILE to that of a full agonist. To monitor how accurately AT_1-[Y292F/N295S] and the chimeras approximate the pharmacodynamics of wild-type AT_1 receptor activation, the effects of SARILE on IP_3 release in the presence and absence of AngII were tested (Figure 6). Our results show that 1 μM SARILE does indeed possess minimal agonist activity at the AT_1 receptor—only 25.7 ± 4.81% of that found for an equivalent concentration of AngII. Furthermore, SARILE was able to partially block receptor activation elicited by submaximal (EC_{50}) doses of AngII (as previously determined in Figures 3 and 5). Not surprisingly, a similar result was obtained for SARILE in the case of AT_1[AT_2 CT], which possesses no substitutions in the transmembrane-spanning domains. However, when substitutions are made to the relevant transmembrane-spanning domains, as is the case with AT_1[AT_2 TM7-CT] and AT_1[AT_2 TM6-CT], SARILE behaved as a full agonist. It appears that TM6 and TM7 of the AT_2 receptor can functionally substitute for the corresponding AT_1 receptor domains in the AngII-induced activation mechanism with minimal differences, but such large domain exchanges can result in significant differences in the responses to synthetic AngII analogues such as SARILE. Like AT_1[AT_2 TM7-CT] and AT_1[AT_2 TM6-CT], the AT_1-[Y292F/N295S] mutant also possesses changes at both of the key amino acid residues in the seventh transmembrane-spanning domain—however, SARILE possessed minimal agonist activity here, similar to the wild-type AT_1 receptor. Thus, even with respect to the functional response evoked by AngII receptor antagonists, AT_1[Y292F/N295S] ap-

proximates the wild-type AT_1 receptor. This strongly supports the idea that the same domains and even the amino acid residue positions mediate AngII-dependent activation in either AngII receptor.

DISCUSSION

The physiological effects of many, if not most, chemokines, neurotransmitters, and hormones, including AngII, are mediated by GPCRs. The breadth of this receptor superfamily underscores an important point—that proteins of dissimilar amino acid sequences may, nonetheless, conform to a similar three-dimensional structure. All GPCRs adopt a seven transmembrane-spanning domain topology: thus, a particular three-dimensional structure need not be derived from or restricted to a single amino acid sequence. Since the functional properties of any given protein are a direct consequence of its three-dimensional structure, it stands to reason that proteins—or protein domains—of different primary sequence yet similar three-dimensional structure may possess similar functional properties as well. The results of this study on the structure−function relationships of AngII receptors strongly support this line of reasoning.

At approximately 34%, the level of homology between the two AngII receptor subtypes is low for GPCRs that bind the same endogenous ligand. Despite the high degree of divergence in sequence, the AT_1 and AT_2 receptors do share some structural and functional similarities: (1) the predicted seven transmembrane-spanning domain topology (*12, 44*); (2) interaction with G proteins at their intracellular faces (*13, 45*); (3) the ability to bind AngII with equal affinity; and (4) recognition of AngII as an agonist and activation of their respective signaling pathways in response to it. In fact, both the AT_1 and AT_2 receptors use the same domain—the amino terminus—to assist in binding AngII with high affinity, despite the absence of any homology between them (*40*), supporting the idea that domains with dissimilar amino acid sequences can have similar functional properties.

The amino acids involved in the intrareceptor activation mechanism immediately following AngII binding have been a focus of study for the AT_1 receptor. In the instances of Asp^{74} in TM2 and Tyr^{292} in TM7, conservative mutations resulted in loss of agonist-induced AT_1 receptor activation, and led to a proposed hypothetical interaction of these two residues in stabilizing the activated state of the receptor (*24, 25*). Both His^{256} and Phe^{259} in TM6 have been shown to be important for the recognition of AngII as an agonist. Conservative substitution of either residue resulted in strongly diminished IP_3 release upon AngII binding (*27*). It was suggested that removal of His^{256} or Phe^{259} from the AT_1 receptor impairs interaction with Phe^8 of AngII, a necessary determinant for agonist activity of the peptide. Other studies have identified residues involved in maintaining the AT_1 receptor in the inactive state in the absence of AngII—an equally important aspect of AngII-dependent activation. Specifically, when Asn^{111} (in TM3) or Asn^{295} (in TM7) was mutated, an approximate doubling of the basal IP_3 level was reported (*28, 29*). Clearly, these studies unequivocally support the importance of residues in TM6 and TM7 of the AT_1 receptor in controlling agonist-dependent activation. It is noteworthy that many of these TM6 and TM7 residues—His^{256}, Phe^{259}, Tyr^{292}, and Asn^{295}—are *not* conserved in the AT_2 receptor. In fact, in some instances (*25, 28*) the residues which are present at these positions in the AT_2 receptor were directly proven inadequate for maintaining normal activation of the AT_1 receptor. Indeed, virtually nothing is known about the activation mechanism of the AT_2 receptor. However, with recent studies proposing a role for the AT_2 receptor in tissue development and remodeling (*34, 35*), interest has increased in its structure/function relationships.

By using carefully constructed chimeric AngII receptors, we tested whether the corresponding domains of the AT_2 receptor could functionally substitute for the important domains (i.e., TM6 and TM7) of the AT_1 receptor in AngII-induced activation, despite the key differences in their respective amino acid sequences. Note that other amino acids in the AT_1 receptor TM6 and third extracellular loop which are essential for AngII binding (*19, 47*) are, fortuitously, conserved in the AT_2 receptor (*36, 48*)—an important rationale for selecting portions of the AT_2 receptor (rather than other GPCRs) to test the veracity and flexibility of the currently proposed AT_1 activation model. Furthermore, by retaining the third intracellular loop of the AT_1 receptor in our chimeras, we were able to measure activation by quantitating IP_3 release, thereby avoiding difficulties previously encountered in deciphering AT_2 receptor signaling (*12, 49−51*). Chimera-based approaches have been used in earlier reports on AngII receptor structure/function (*23, 30*). Our objective here was to have an easily quantifiable index of receptor activation, rather than exploring effector coupling domains or signaling pathways. While the particular signaling pathway measured here (phosphoinositide hydrolysis) is more commonly associated with the AT_1 receptor, in the context of quantifying chimera activation it allows us to extend our observations to consider activation determinants of either AngII receptor subtype.

The results of the assays performed on the chimeric AT_1/AT_2 receptors show the ability both to bind AngII as well as to activate phosphoinositide hydrolysis. This is surprising since previous studies utilizing the aforementioned point mutants showed that the corresponding AT_2 TM7 residues disrupt AngII-dependent activation of the AT_1 receptor (*25, 28*). The current model would predict either constitutively activated chimeras or, more likely, chimeras that fail to activate IP_3 release upon AngII stimulation. This is especially true for AT_1[AT_2 TM6-CT], which additionally lacks the AT_1 TM6, and therefore lacks His^{256} and Phe^{259}—the important residues for recognizing AngII as an agonist (*27*). The residue in AT_1[AT_2 TM6-CT] corresponding to His^{256} is a Phe, and the residue corresponding to Phe^{259} is a Leu. Perhaps the Phe for His^{256} substitution is sufficiently conservative to preserve normal agonist activation (there are no previous studies recommending that the corresponding Phe in AT_2 is disruptive). Both residues are aromatic, and the stabilizing interaction of $\pi-\pi$ electrons (*52*) between these residues and Phe^8 of AngII may be the crucial activation trigger. However, the substitution of Phe^{259} with Leu is hardly conservative. It poses questions about how AT_1[AT_2 TM6-CT] can recognize AngII as an agonist and activate, when even the conservative mutation of Phe^{259} to a Tyr (*27*) strongly diminishes activation. The current model would have predicted that AT_1-[AT_2 TM6-CT] be especially unresponsive to AngII.

Yet AT_1[AT_2 TM6-CT], like AT_1[AT_2 CT] and AT_1[AT_2 TM7-CT], activates in an AngII-dependent manner, all with

EC_{50} values similar to the AT_1 receptor. AT_1[AT_2 TM7-CT] possesses some constitutive activation, which explains its apparently enhanced efficacy relative to the others. It is unlikely, however, that the partial constitutive activation is simply due to the substitution of Asn^{295} with Ser, as the study by Balmforth et al. (*28*) might suggest; this very substitution is also made in AT_1[AT_2 TM6-CT] with no effect on basal IP_3 levels. The natural conclusion from the results of the chimeras is that similar function may exist in domains with dissimilar amino acid sequences. The AT_1 receptor must possess a limited flexibility with respect to some of the structural elements (i.e., TM7 and TM6) involved in agonist-induced activation.

Our results clearly show that the variation in expression levels of the mutants has little to no discernible impact on their maximal functional efficacies (although the mutations could themselves have dramatic effects, e.g., AT_1[AT_2 TM7] and AT_1[Y292F]). It is not uncommon in transfected cell systems for mutant expression levels to vary from wild-type levels; furthermore, the common occurrence of so-called "spare receptors" (that population of the receptors expressed that exceeds the cell's capacity to functionally respond to cognate agonist, yet are still detectable in radioligand binding assay) can affect the proportionality of B_{max} and maximal functional efficacy (*43*, *56*, *57*). The potential for incongruencies between binding and function underscores the need to do both kinds of experiments in any meaningful evaluation of the effect(s) of receptor mutations. For this reason, we thoroughly characterized the functionality (maximal efficacy, dose−response relationship, pharmacodynamics) of each mutant receptor and made direct comparisons to the wild type. That the mutant receptors in this study often possessed any agonist-induced activation, let alone activation that was comparable to the wild type, is very compelling.

It is also noteworthy that the presence of the AT_1 cytoplasmic tail was not necessary for any of the chimeras to stimulate phosphoinositide hydrolysis. This has been a matter of some debate in the field: while Ohyama et al. (*13*) originally reported that a portion of the cytoplasmic tail aids in the coupling of the AT_1 receptor to G_q, a more recent study (*23*) demonstrated that the third intracellular loop of the AT_1 receptor is alone sufficient for G_q coupling. Our results support the latter finding.

The lack of phosphoinositide hydrolysis upon AngII treatment of AT_1[AT_2 TM7] is puzzling. Saturation binding results confirm that AT_1[AT_2 TM7] binds AngII with K_D and B_{max} values in the range of other chimeras which *do* activate phosphoinositide hydrolysis. The reason behind the lack of signaling by AT_1[AT_2 TM7] is presently unclear. Small changes at transmembrane-spanning domains reportedly can disrupt interactions with G proteins (*53*), although guanine nucleotides can reduce agonist binding to AT_1[AT_2 TM7], suggesting it still couples to a G protein. The lack of signaling could be attributable to a unique conformational twist in AT_1[AT_2 TM7] which causes specific uncoupling from G_q but not other associated G proteins (e.g., G_i). Alternatively, the AT_2 TM7 may require adjacent regions of the AT_2 cytoplasmic tail in order to adopt the proper conformation for AngII-induced activation to occur. While more experimentation is needed to determine why AT_1[AT_2 TM7] behaves differently from the other chimeras, its lack of a functional response does not detract from the results obtained with the other chimeras or the conclusions drawn from them regarding structure and function.

Of all the domains examined, the ability of the AT_2 TM7 to be functionally similar to the AT_1 TM7 in mediating activation seems especially contradictory to earlier findings: activation was disrupted in one way or another after the key residues in TM7 of the AT_1 receptor (Tyr^{292} or Asn^{295}) were substituted with the corresponding residues of the AT_2 receptor (Phe^{308} or Ser^{311}, respectively). There are two possible explanations for these seemingly paradoxical findings. The first is that Phe^{308} and Ser^{311} are dependent on the local structural environment of the AT_2 TM7 to function properly in activation. The influence of local structure is not unprecedented, and has already been shown for Asn^{111}: this residue is important in maintaining the AT_1 receptor in an inactive state prior to AngII binding (*29*), and it *is* conserved in the AT_2 receptor. Yet in another study, replacement of TM3 of the AT_1 receptor with that of the AT_2 receptor resulted in constitutive activation, even though Asn^{111} had been, in effect, preserved (*30*). Proper participation of this Asn in receptor activation was impaired by the effects of the different local structural context. We can extend this line of reasoning to the substituted Phe or Ser from TM7 of the AT_2 receptor: both have reportedly failed to preserve AngII-induced activation when placed in the local structural context of the AT_1 TM7; yet, in our study, they preserve receptor activation when kept within the context of the AT_2 TM7. The other possible explanation for our paradoxical findings is that our chimeras preserve activation because they effectively create substitutions at *both* loci simultaneously. It is possible the abberation in AT_1 activation created by replacement of Tyr^{292} with Phe is somehow corrected by the additional replacement of Asn^{295} with Ser (and vice-versa), irrespective of the local structural context. The effects of simultaneous replacement at the two loci had never been tested before.

To clarify the issue, we tested whether normal activation of the AT_1 receptor would be preserved in a more focused AT_1[Y292F/N295S] double-point mutant. If the context of the AT_2 TM7 were the crucial factor enabling Phe^{308} and Ser^{311} to preserve AngII-dependent receptor activation in the chimeras, the AT_1[Y292F/N295S] mutant should not activate. If, however, the simultaneous substitution of this *pair* of residues were the critical factor in preserving activation, then AT_1[Y292F/N295S] might function normally. Our study shows that, while a single AT_1[Y292F] point mutant does indeed show impaired activation, AT_1[Y292F/N295S] activates in a manner very similar to the AT_1 receptor with respect to efficacy and EC_{50}. Interestingly, the single mutation of Asn^{295} to Ser only blunted AngII-induced IP_3 release, and did *not* induce the originally reported (*28*) constitutive activation. Reports by others have also failed to observe constitutive activation associated with this particular mutation (*30*, *54*, *55*). This calls into question the involvement of Asn^{295} in maintaining the AT_1 receptor inactive. Indeed, it suggests that Asn^{295} is more involved in formation of the activated state based on the functional results of both the AT_1[N295S] (blunted activation) and AT_1[Y292F/N295S] (restored activation) mutants.

In fact, AT_1[Y292F/N295S] more accurately preserved functionality of the AT_1 receptor in ways that most of the chimeras did not. SARILE was a weak agonist at both the

wild-type AT_1 receptor and AT_1[Y292F/N295S]; yet it is a full agonist at AT_1[AT_2 TM7-CT] and AT_1[AT_2 TM6-CT]. Substitution of transmembrane-spanning domains from the AT_2 receptor into the AT_1 receptor probably weakens or eliminates some unidentified interhelical interactions ordinarily holding the receptor inactive, such that SARILE possesses the sufficient pharmacophores to activate these chimeras. For example, while SARILE lacks Phe^8, it still possesses Arg^2, which is also necessary for full agonism of the AngII peptide (*20*). Perhaps, given the structural arrangement of the chimeras, Arg^2 is also now sufficient for full agonism from these peptides. AT_1[Y292F/N295S], being a less drastic mutation, presumably preserves more of the native interactions stabilizing the inactive receptor, so its pharmacodynamics are similar to those of the wild-type AT_1 receptor. While the precise nature of how two individually disruptive point mutations can together restore normal activation remains to be determined, these results underscore a limitation of individual point mutation-based strategies in determining the full functional significance of amino acid residues. Likewise, the overall results draw attention to the potential limitation of considering only amino acid sequence homology when postulating the functional capabilities of protein domains.

In summary, our study has given both (i) cause to reconsider the stringency of the structural requirements of the mechanism of AT_1 receptor activation, as well as (ii) an initial glimpse at the residues of the AT_2 receptor that may mediate its AngII-induced activation. Despite extensive amino acid dissimilarities, the AT_2 TM6 and TM7 can function similar to the AT_1 TM6 and TM7 in receptor activation. Our data propose that both pairs of TM7 residues—Tyr^{292} and Asn^{295} in the AT_1 receptor, and Phe^{308} and Ser^{311} in the AT_2 receptor—are keystone residues for the ultimate three-dimensional conformation of TM7 in both receptor subtypes, and appear to be interchangeable in the AT_1 receptor. In order for this domain—in either subtype—to have proper three-dimensional conformation for AngII-induced activation, those residues may need to be present in the combinations specified. The single-point mutants therefore disrupt normal activation, but our chimeras and the double-point mutant behave similar to the wild-type AT_1 receptor. The ability to interchange important domains, and even pairs of residues, located in corresponding positions in the two receptor subtypes without disrupting AngII-dependent activation gives reason to hypothesize that a similar activation mechanism may yet be employed by these two very different AngII receptor subtypes.

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