A model of the angiotensin AT₁ receptor and site-directed mutagenesis were used to identify key residues involved in ligand binding. Receptors were stably expressed in human embryonic kidney 293 cells, and their binding properties compared. Wild type receptors exhibited low and high affinity binding sites for peptides. Substitution of Asn¹¹¹, situated in the third transmembrane helix, resulted in a significant alteration in ligand binding with only high affinity binding of the peptides, angiotensin II and angiotensin III. AT₁ receptor selective non-peptide antagonist losartan. From our model it was apparent that Asn¹¹¹ was in close spatial proximity to Asn²⁹⁵ in the seventh transmembrane helix. Substitution of Asn²⁹⁵ produced identical changes in the receptor’s pharmacological profile. Furthermore, the Ser¹¹¹AT₁ and Ser²⁹⁵AT₁A mutants did not require the association of a G-protein for high affinity agonist binding. Finally, the Ser²⁹⁵AT₁A mutant maintained higher basal generation of inositol trisphosphate than the wild type, indicating constitutive activation. We propose that substitution of these residues causes the loss of an interaction between transmembrane helices III and VII, which allows the AT₁ receptor to “relax” into its active conformation.

The renin-angiotensin system plays a vital role in the regulation of cardiovascular function, and its activity may be abnormal in a number of disease states. The effector molecule of these receptors is angiotensin II and the natural ligand Ang II and angiotensin III (Ang III). AT₂ receptors bind Ang II and Ang III with equal affinity (5–8), while AT₁ receptors exhibit a slightly higher affinity for Ang II, between 3- and 20-fold greater than Ang III (9–11). Many of the recognized actions of Ang II appear to be mediated through AT₁ receptors, predominantly through activation of the phospholipase C signal transduction pathway.

AT₁ receptors have been cloned from a variety of species and tissues (9–15). All these AT₁ receptors consist of a single polypeptide, 359 amino acids in length, arranged with a topology comprising seven α-helical transmembrane regions (TM I–VII), typical of the G-protein-coupled receptor family. They display a high degree of sequence identity at the amino acid level (over 94% identical between all mammalian species). Two closely related isoforms (AT₁A and AT₁B) encoded by different genes have been identified in the rat and mouse (13, 16). Recently AT₂ receptors have been cloned (17–19), which are also members of the G-protein-coupled receptor family. They consist of 363 amino acids and share only 34% sequence identity at the amino acid level to the AT₁ receptors.

Mutagenesis studies of the AT₂ receptor have recently identified residues important in the binding of the non-peptide antagonist, losartan, and the natural ligand Ang II. These residues appear to be mutually exclusive, since amino acid substitutions, which affect losartan binding, do not alter Ang II binding, and vice versa. The major determinants of losartan binding appear to be residues located within the transmembrane helices III–VII (20–23). In contrast, the major determinants of Ang II binding appear to be residues present in the extracellular regions, particularly in the N-terminal extension adjacent to the first transmembrane helix and in the C-terminal part of the third extracellular loop (24, 25). However, a conserved residue, Lys¹⁹⁹ in the fifth transmembrane helix (TM V) of the AT₁ receptor, has recently been reported to be crucial for the binding of both peptide and non-peptide ligands (26). In the present study we constructed a three-dimensional model of the AT₁A receptor using the method of Donnelly et al. (27) to guide site-directed mutagenesis aimed at identifying potential residues involved in ligand binding. The consequences of substituting either Asn¹¹¹, in the third transmembrane helix (TM III), or Asn²⁹⁵, in the seventh transmembrane helices (TM VII), on ligand binding and receptor coupling are presented. These data suggest that a specific interaction between TM III and TM VII may be a major determinant of AT₁ receptor isomerization from an inactive to active conformation.
EXPERIMENTAL PROCEDURES

Materials—Ang II, Ang III, bacitracin, bovine serum albumin (pro-
tease-free), GTP-γ-S, and fura 2-AM were purchased from Sigma. 125I-
[Sar1,Ile8]angiotensin II and 125I-angiotensin II from DuPont NEN.
PnNH2F6AII and CGP42112A from Bachem. Losartan (DuP 753) was
genously donated by Glaxo Group Research Limited. Hygromycin B was
Calbiochem, Transf-
tam from Integra Biosciences Ltd., and Mutagenie Phagemid In Vivo
utagenesis Version 2 kit from Bio-Rad. pBluescript KS+ and M13K07
help phage was from Stratagene Ltd. and pCEP4 and Escherichia coli
CJ236 cells were purchased from Invitrogen. The CDNA clone-encoding
rat AT1 receptors was kindly provided by Glaxo Group Research Limited.

Three-dimensional Modeling—Modeling was performed according to
the method of Donnelly et al. (27). Briefly, following sequence alignment
of the rat AT1A receptor to the human β2-adrenergic receptor, the seven
identified α-helices were constructed using the interactive graphics
program InsightII (Biosym Technologies). The projection map of bovine
rhodopsin was used to position these helices into a three-dimensional
helical bundle, the order of which was assumed to be analogous to the
arrangement in bacteriorhodopsin. The internal faces and relative
depth of each helix in the bilayer were deduced by Fourier transform
analysis. The connecting intracellular and extracellular loops are not
included in the model due to the lack of structural information.

Site-directed Mutagenesis—The entire coding region of the rat AT1A
receptor (in vitro fragment of 2 kilobases) was subcloned into the dIII
poly linker sites of pBluescript KS+, and single-stranded DNA was
rescued from CJ236 cells using M13K07 helper phage. Mutagenesis
was performed with a Mutagenie Phagemid In Vivo Mutagenesis
Version 2 kit and confirmed by dyeexy sequencing using Sequenase II
(U. S. Biochemical Corp.). The wild type and mutated AT1A cDNAs were
subcloned into the mammalian expression vector pCEP4 using the restric-
tion enzymes RadiIII and NotI.

Permanent Expression of Receptors in HEK293 Cells—HEK293 were
cultured in modified Eagle’s medium containing Earle’s salts, supple-
mented with 10% (v/v) fetal calf serum, 1% nonessential amino acids, 50
µg/ml gentamicin, 100 units/ml penicillin G, 100 µg/ml streptomycin,
and 0.25 µg/ml amphotericin at 37 °C in a humidified atmosphere of
airCO2 (19:1). Cells were transfected with 12 µg of pCEP4 containing
either the cDNA for the wild type or mutated AT1A receptors using
Transfectam, according to the manufacturer’s instructions. Stable
expression of AT1A receptors in HEK293 cells was achieved by addition
of 200 µg/ml hygromycin B to the medium 3 days after transfection and
for all subsequent passages of the cells.

Binding Assay—Preparation of transfected HEK293 cell membranes
and subsequent radioligand-receptor binding assays were undertaken as
described previously (28) with the following modifications. All com-
petitors were diluted in assay buffer which consisted of 100 mM NaCl,
20 mM Hepes (pH 7.4), 1 mM EDTA, 10 mM MgCl2, bacitracin (0.1% w/v),
and bovine serum albumin (0.1% w/v). Nine to 11 concentrations of
competitor were examined, each concentration in duplicate. Mem-
branes were incubated with 125I-[Sar1,Ile8]angiotensin II and competi-
tors for 60 min at room temperature. In the experiment to determine
the effect of GTP-γ-S on Ang II binding, the total reaction volume was
500 µL, consisting of 200 µL of membranes (10–50 µg of protein), 50 µL of
125I-angiotensin II (1.2 nM), and 50 µL of GTP-γ-S (60 µM) or assay buffer.

Measurement of Intracellular Calcium in Transfected HEK293 Cells—Cells
were subcultured on glass coverslips and used at confluence. The coverslips
were incubated with the cell permeant fluores-
cent Ca2+probe fura2-AM (5
M) in a Krebs-Ringer buffer (pH 7.4) consisting of
145 mM NaCl, 5 mM KCl, 1.3 mM MgCl2, 1.2 mM NaH2PO4, 1.3 mM
CaCl2, 10 mM glucose, 20 mM Hepes, at 37 °C for 1 h. Loaded cells were
then washed free of extracellular dye, and the coverslip was mounted in an
open culture chamber which was then placed on the stage of an
inverted Nikon microscope and maintained at 37 °C in Krebs-Ringer
buffer. Dynamic video imaging was carried out as described previously
(29), using a digital camera and TARDIS software (Applied Images,
Sunderland, UK) and a low light level intensified charged coupled
department (Photonic Science, Robertsbridge, UK).

Measurement of Inositol 1,4,5-Trisphosphate (IP3) Production in
Transfected HEK293 Cells—Cells were subcultured on 30-mm Petri
plates and used at confluence. Each dish was washed with assay
medium (inositol-free Dulbecco’s modified Eagle’s medium containing
20 mM Hepes, 0.25% (w/v) bovine serum albumin, 0.25% (w/v) bac-
tracin, pH 7.4; three times at 2 ml each). After washing, 1 ml of medium
containing either no reagents or 100 nM angiotensin II was added, and
incubations were performed for 15 s. The assay was stopped by the
addition of ice-cold 20% trichloroacetic acid (0.5 ml). Subsequent
extraction and measurement of IP3 production was undertaken using an
inositol 1,4,5-trisphosphate (IP3) radioreceptor assay kit according to
the manufacturer’s instructions. Functional studies for both wild type and
Ser295AT1A receptors were routinely performed in a single assay.

Data Analysis—Radioligand binding results are expressed as a per-
centage of the control (i.e. specific binding in the absence of any com-
petitor). The binding data were analyzed and IC50 values determined by
non-linear regression analysis using GraphPad Prism (GraphPad Soft-
ware, San Diego, CA). Competition curves were simultaneously fit to
one- and two-site models, and an F test was used to determine the
most appropriate model. All IC50 values are quoted as the mean ± S.E.

RESULTS

Characterization of the Wild Type AT1A Receptor—The wild

ype AT1A receptor stably expressed in HEK293 cells was char-
acterized by examining the ability of ligands to compete with
0.1 nM 125I-[Sar1,Ile8]angiotensin II binding to membrane prep-
arrations (Fig. 1A and Table I) and the ability of Ang II to
stimulate a rise in intracellular calcium (Fig. 2A). GraphPad
Prism Analysis of the Ang II, Ang III, and pNH2F6AII competition
curves fitted best a two-site model. These ligands com-
peted for both high and low affinity binding sites, with esti-

ated IC50 values (nm) of 0.8 ± 0.1, 3.5 ± 0.2, and 63 ± 30 for high,
and 12 ± 2.8, 120 ± 12, and 5180 ± 4075 for low affinity
binding sites, respectively. The AT2 selective non-peptide an-
tagostant losartan competed for a single high affinity binding
site (IC50 value of 26.1 ± 1.5 nm), while the AT2 selective
ligands, CGP42112A, and PD123319 (an analogue of
PD123177) competed for single low affinity binding sites (IC50
values of 5930 ± 1466 and 102650 ± 6582 nm, respectively).
The functional coupling of the receptor was demonstrated by
measuring intracellular calcium. Addition of Ang II (100 nm) to
transfected cells resulted in a rapid, transient increase in in-
tracellular calcium, which reached maximum levels by 5–10 s
and then declined toward resting levels by 60 s (Fig. 2A).
Neither this response nor specific binding of the radioligand
125I-[Sar1,Ile8]angiotensin II was observed in untransfected
cells (data not shown).

Characterization of the Ser111 and Ala111 Mutant AT1A Re-
ceptors—The replacement of an asparagine residue (Asn111),
located in the third transmembrane helix, with a serine, re-
sulted in both subtle and large changes in the binding affinities
of the AT1- and AT2-selective ligands studied here (Fig. 1B
and Table I). In contrast to the findings with the wild type receptor,
all of the ligands now competed for binding to a single site.
Of particular interest was the observation that the resulting IC50
values for Ang II, Ang III, and pNH2F6AII (0.9 ± 0.1, 0.6 ± 0.1,
and 68 ± 13.6 nm, respectively) were very similar to the IC50
values recorded for the binding of these ligands to their high
affinity sites displayed by the wild type receptor. One con-
sequence of this altered binding was that the mutated receptor
now had equal affinity for Ang II and Ang III; an observation
typical of an AT2 receptor. Furthermore, the AT2 selective
peptide ligand, CGP42112A, also displayed an increased bind-
ing affinity (28 fold), in keeping with this trend toward AT2
receptor pharmacology. However, the AT2 selective non-pep-
tide ligand PD123319 showed only a modest 3-fold increase in
binding affinity for the mutated receptor.

In contrast to the above increases in binding affinity, the
ability of the specific AT2 non-peptide antagonist losartan to
bind to the mutated receptors was severely impaired (a 148-
fold decrease in binding affinity). The rank order of potency
was thus Ang III = Ang II > pNH2F6AII > CGP42112A >
losartan > PD123319. The pharmacological profile of the
Ala$^{111}$AT$_{1A}$ receptor was identical to that of the Ser$^{111}$AT$_{1A}$ receptor, with only minor differences in the IC$_{50}$ values for all the ligands tested (Fig. 1C and Table I). Addition of Ang II (100 nM) to HEK293 cells transfected with the two Asn$^{111}$ mutant AT$_{1A}$ receptors resulted in a rapid, transient increase in intracellular calcium, similar to that observed in HEK293 cells transfected with the wild type receptor (Fig. 2, B and C).

Characterization of the Ser$^{295}$AT$_{1A}$ Receptor—Analysis of the molecular model of the AT$_{1A}$ receptor revealed that Asn$^{111}$ located in the third transmembrane helix was in spatial proximity to Asn$^{295}$ in the seventh transmembrane helix (Fig. 3, A and B), raising the possibility of an interaction between these two residues. Since this interaction may be disrupted by the mutation of Asn$^{111}$ to either an alanine or serine residue, its loss may be responsible for the observed changes in ligand binding. This hypothesis was tested by creating a Ser$^{295}$AT$_{1A}$ receptor. The pharmacological profile of the Ser$^{295}$AT$_{1A}$ receptor was identical to that of the Asn$^{111}$ mutants, with only minor differences in the IC$_{50}$ values for all the ligands tested (Fig. 1D and Table I). Furthermore, addition of Ang II (100 nM) to HEK293 cells transfected with the Ser$^{295}$AT$_{1A}$ receptor resulted in a rapid, transient increase in intracellular calcium, similar to that observed in HEK293 cells transfected with the wild type receptor (Fig. 2D).

**Effect of GTP$^\gamma$S on Angiotensin II Binding**—The effects of GTP$^\gamma$S on $^{125}$I-angiotensin II binding were examined in membranes prepared from HEK293 cells expressing wild type, Ser$^{111}$AT$_{1A}$, and Ser$^{295}$AT$_{1A}$ receptors (Fig. 4). GTP$^\gamma$S reduced Ang II binding to the wild type receptor to 13.8 ± 0.4% of that in the absence of GTP$^\gamma$S (n = 3), whereas it had no effect on Ang II binding to Ser$^{111}$AT$_{1A}$ (98.6 ± 2.1%, n = 3) or Ser$^{295}$AT$_{1A}$ (102.7 ± 3%, n = 3) receptors. This suggests that,

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**Table I**

| Ligand    | Wild type AT$_{1A}$ | Ser$^{111}$AT$_{1A}$ | Ala$^{111}$AT$_{1A}$ | Ser$^{295}$AT$_{1A}$ |
|-----------|----------------------|----------------------|----------------------|----------------------|
| Ang II    | 0.8 ± 0.3 (38%)      | 0.9 ± 0.1            | 1.2 ± 0.2            | 1.6 ± 0.1            |
| Ang III   | 3.5 ± 0.2 (56%)      | 0.6 ± 0.1            | 1.0 ± 0.3            | 0.9 ± 0.1            |
| Losartan  | 120 ± 12             | 3867 ± 570           | 6467 ± 521           | 5400 ± 306           |
| CGP42112A | 12.0 ± 2.8           | 5930 ± 1466          | 210 ± 56             | 106 ± 9              |
| pNH$_2$F$_6$AII | 63.4 ± 30.3 (22%) | 68 ± 14              | 26.6 ± 11.9          | 30 ± 2.4             |
| PD123319  | 51810 ± 4075         | 102650 ± 6582        | 34500 ± 1060         | 25133 ± 2826         | 32600 ± 2170         |
in contrast to the wild type receptor, coupling to G-proteins is not required for these mutated receptors to maintain their high affinity binding.

**Inositol 1,4,5-Trisphosphate Production in Transfected HEK293 Cells—** IP₃ production was examined in HEK293 cells expressing either wild type AT₁A or Ser295AT₁A receptors (Fig. 5). Even though the level of expression of the Ser295AT₁A receptor (1385 ± 123 fmol/mg total cell protein, n = 3) was less than that of the wild type AT₁A (1990 ± 178 fmol/mg total cell protein, n = 3), the cells expressing the Ser295AT₁A receptor exhibited higher levels of agonist-independent (basal) production of IP₃ (2.2 ± 0.4-fold higher than wild type receptor basal value, n = 6; Fig. 5, left). Furthermore, although 100 nM Ang II stimulated both wild type AT₁A and Ser295AT₁A receptors, the total IP₃ production by cells expressing the Ser295AT₁A receptor was lower (64 ± 7% of the wild type AT₁A, n = 6) than that produced by cells expressing the wild type AT₁A receptor (Fig. 5, right).

**DISCUSSION**

Using our model of the rat angiotensin AT₁A receptor, we selected to mutate the asparagine residue at position 111, located in TM III. Substitution with either a serine or alanine residue resulted in a marked alteration in the binding of several peptides and the AT₁ selective non-peptide ligand losartan. Importantly, only high affinity binding was observed following Asn¹¹¹ substitutions, in contrast to the wild type receptor, which displayed both high and low affinity binding for Ang II, Ang III, and pNH₂F₆AII. Furthermore, the binding affinity of the AT₁ receptor-selective non-peptide antagonist losartan decreased (148–248-fold). Identical results were obtained when we substituted a serine residue for an asparagine residue in TM VII (Asn²⁹⁵), identified by our three-dimensional model of the rat AT₁A receptor as being in spatial proximity to Asn¹¹¹. All these mutations retained Ang II mobilization of intracellular calcium, indicating that these substitutions did not perturb coupling of these receptors to the phospholipase C signal transduction pathway.

The binding of agonists, but not antagonists, to G-protein-coupled receptors in membrane preparations is characterized by high and low affinity binding states. The high affinity state is believed to be the active conformation of the receptor, consisting of a ternary complex involving the agonist, the receptor, and the G-protein, while the low affinity state is believed to be an inactive binary complex of agonist and receptor (30). In the presence of guanine nucleotides, which cause dissociation of G-proteins from their receptors, the high affinity state appears to be converted into the low affinity state (31).

Ang II receptors are typical of G-protein-coupled receptors, existing in high and low affinity agonist binding states in various tissues, the higher being converted to the lower in the presence of guanine nucleotides (32–35). We have also observed that wild type recombinant AT₁A receptors display both high and low affinity agonist binding states for Ang II. Furthermore, using a low concentration of ¹²⁵I-angiotensin II, which would preferentially bind to the high affinity state, we demonstrated that this high affinity state was lost when binding was undertaken in the presence of the nonhydrolyzable guanine nucleotide GTPγS. This suggested that the high affinity state was also dependent on formation of a ternary complex involving Ang II, the AT₁A receptor, and a G-protein.

Ang III and pNH₂F₆AII also bound to high and low affinity states of the wild type AT₁A receptor, suggesting both are agonists. While Ang III is accepted as an agonist, there are no direct reports of the agonist actions of pNH₂F₆AII. We included...
pNH₂F₆AII in our studies since it had been reported that it preferentially bound to AT₂ receptors and might therefore be useful as a subtype selective ligand (4). However, in keeping with our observations of its agonist properties, these authors also reported that the potency of binding of pNH₂F₆AII to AT₁ receptors was diminished in the presence of GTPγS but did not attempt to estimate the high and low affinity agonist binding state values (4).

There was a striking similarity between the estimated high affinity agonist binding state values for Ang II, Ang III, and pNH₂F₆AII displayed by the wild type AT₁A receptor and their corresponding single site binding affinities in the Asn₁¹¹ and Asn₂⁹⁵ AT₁A receptors. This suggests that a consequence of these substitutions is to allow all of the receptors expressed to adopt the high affinity agonist binding state conformation. However, the effects of GTPγS on Ang II binding indicate that, whereas the wild type receptor requires the association of a G-protein to form the ternary complex to adopt the conformation of a high affinity agonist binding state, the Ser₁¹¹AT₁A and Ser₂⁹⁵AT₁A mutants do not (Fig. 4). While, in many instances, the lack of a GTPγS effect on agonist binding merely indicates that a mutant receptor is no longer coupled, this was clearly not the case here. All three mutants produced transient increases in intracellular calcium similar to the wild type receptor. Thus substitutions at Asn₁¹¹ and Asn₂⁹⁵ produce a G-protein-independent, high affinity agonist binding state conformation of the AT₁A receptor which is still capable of coupling to a G-protein. Such features are characteristic of constitutively active receptors.

In a subsequent experiment (Fig. 5) we compared IP₃ production in cells expressing either wild type AT₁A or Ser₂⁹⁵AT₁A receptors. In agreement with the intracellular calcium data we confirmed that the Ser₂⁹⁵AT₁A receptor was capable of coupling to a G-protein. However, we also noted two discrepancies between the cells expressing the wild type AT₁A and Ser₂⁹⁵AT₁A receptors. First, although the level of expression of the Ser₂⁹⁵AT₁A receptor was less than that of the wild type AT₁A receptor, cells expressing the Ser₂⁹⁵AT₁A receptor exhibited higher levels of agonist-independent production of IP₃ (approximately 2-fold higher basal), suggesting that this receptor is constitutively active. Second, Ang II stimulated IP₃ production by cells expressing the Ser₂⁹⁵AT₁A receptor was lower than that produced by cells expressing the wild type receptor. Since the Ser₂⁹⁵AT₁A receptor appears constitutively active, the underlying mechanism may be constitutive desensitization, as previously reported for a constitutively active β₂-adrenergic receptor (36). Similar findings are anticipated for the Asn₁¹¹ mutants, but so far have not been undertaken.

Constitutive activation of a G-protein coupled receptor was first described in the α₁B-adrenergic receptor (37), with substitution of amino acids in the C terminus of the third intracellular loop resulting in increases in both agonist binding affinity and basal inositol phosphate production (suggesting receptor activation in the absence of an agonist). Mutations that cause constitutive activation appear to occur at widely distributed sites in the sequences of G-protein-coupled receptors (38). However, relevant to our work is the observation that substitution of Glu₁¹³ in TM III or Lys₂⁹⁶ in TM VII cause constitutive activation of rhodopsin (39). According to our model, equivalent residues to these in the AT₁A receptor would be positioned two helical turns and one helical turn above Asn₁¹¹ and Asn₂⁹⁵, respectively. Of particular interest is that substitution of Cys₁²⁸ in the α₁B-adrenergic receptor leads to constitutive activation (40). This residue is equivalent in position to Asn₁¹¹ in the AT₁A receptor and both receptors are coupled to phospholipase C.

Previous approaches to identifying residues important in the binding of ligands to AT₁ receptors have, in general, been undertaken comparing the effects of substituting residues on the binding of non-peptide antagonists (s) and at most only one or two peptide ligands. Although such studies have established that peptides bind differently to non-peptide Ang II receptor...
Membranes were prepared from HEK293 cells stably expressing wild type AT$_{1A}$, Ser$^{111}$AT$_{1A}$, and Ser$^{295}$AT$_{1A}$ receptors, and the binding of 0.2 nM $^{125}$I-angiotensin II to the membranes was measured in the presence and absence of 10 µM GTP·S as described under "Experimental Procedures." The data are expressed as the percentage of the specific binding measured in the absence of GTP·S and are shown as means ± S.E. from three independent experiments, each performed in triplicate.

antagonists (20–24), the limited range of ligands used may have resulted in inappropriate conclusions. For example, Schambye et al. (21) reported that substitution of Asn$^{295}$ with a serine residue substantially reduced non-peptide antagonist binding without affecting the binding of Ang II or [Sar$^1$,Leu$^8$]angiotensin II. They concluded that this residue was directly or indirectly interacting with the non-peptide antagonists, but was not involved in the binding of peptides. Recently, similar results and conclusions were reported independently by two groups (22, 23) for Asn$^{111}$. However, although the present study confirms some of the above observations, our alternative approach suggests a very different conclusion regarding the function of these two residues.

We determined which residues to mutate using our three-dimensional model and chose to explore a wide series of peptides and non-peptide antagonists to test predictions from the model. These included peptides that display differential binding to AT$_1$ and AT$_2$ receptors. Our findings indicate a pivotal role for the asparagines at positions 111 and 295 in the isomerization step described in the allosteric ternary complex model (41). In this model it is proposed that the receptor undergoes an isomerization from an inactive (R form) to an active (R* form) state which couples to G-proteins (R*G). This isomerization involves conformational changes which may occur spontaneously, or be induced by agonists or appropriate mutations which abrogate the normal 'constraining' function of the receptor, allowing it to “relax” into the active conformation. The identical effects of mutating Asn$^{111}$ in TM III and Asn$^{295}$ in TM VII on the binding of a series of agonists and antagonists, suggests that these two Asn residues may have a complementary role in the receptor isomerization. Due to the potential spatial proximity of these two residues identified by our three dimensional model (Fig. 3, A and B), this role may be through direct hydrogen bonding. Substitution of a serine residue would be expected to reduce and alanine to eliminate, hydrogen bonding associated with the asparagine residues. Thus one of the major determinants of the isomerization of the AT$_{1A}$ receptor from the R to R* form may be the breaking of hydrogen bonding between Asn$^{111}$ in TM III and Asn$^{295}$ in TM VII.

Inverse agonists are ligands which are postulated to possess a higher affinity for the inactive R form of the receptor when compared to the active R* and R*G forms and are capable of shifting the equilibrium from R*G by stabilizing the R conformation (for review, see Milligan et al. (42)). In the present study, the marked reduction in the binding affinity of losartan observed following substitution of either Asn$^{111}$ or Asn$^{295}$, raises the possibility that it is not an antagonist, but an inverse agonist.

A further interesting but more speculative implication of the present study is that the interaction between Asn$^{111}$ and Asn$^{295}$ may be fundamental in determining the apparent peptide binding selectivity of AT$_1$ and AT$_2$ receptors. The pharmacological profile of the mutant receptors, particularly of the peptides, was closer to that of an AT$_2$ receptor (5–8), than an AT$_1$. Only the change in the affinity of CGP42112A is not entirely consistent with the typical AT$_2$ binding of peptides. This discrepancy may be due to the fact that CGP42112A also contains non-peptide modifications and thus is quite unlike Ang II, Ang III, or pNH$_2$F$_6$AII and therefore would be less likely to share similar "peptide" binding epitopes. Furthermore, in addition to displaying similar peptide binding affinities, the binding of Ang II to AT$_2$ receptors is also unaffected by the presence of guanine nucleotides (4, 6, 35, 43) as observed here for the mutant receptors. Finally, the cloning of the AT$_2$ receptor has now revealed that it also has an asparagine residue in TM III at an equivalent position to Asn$_{111}$ of the AT$_1$ receptor. However, in TM VII of the AT$_2$ receptor, a serine residue corresponds to the asparagine residue here for the mutant receptors. Finally, the cloning of the AT$_2$ receptor.
Asn111 in TM III or Asn295 in TM VII leads to an identical amino acid sequence corresponding to transmembrane helices III and VII of rat and human AT1 and AT2 receptors, as deduced from their cDNAs. Boxed amino acids correspond to Asn\textsuperscript{111} and Asn\textsuperscript{295} of the rat AT\textsubscript{1}A receptor, respectively.

In summary, we have established that substitution of either Asn\textsuperscript{111} in TM III or Asn\textsuperscript{295} in TM VII leads to an identical conformational change in the receptor. The conformation adopted appears to be the active (R\textsuperscript{2}) form of the receptor. The spatial proximity of these two residues suggests that the mechanism responsible for this conformational change may be the breaking of a hydrogen bond interaction between these two residues. The proximity of these two helices has been postulated by us and others in three-dimensional models based upon the projection map of bovine rhodopsin (27, 44, 45). Earlier models based upon the bacteriorhodopsin structure tend to result in TM III and TM VII being more distant (46). To our knowledge, this is the first evidence of a direct interaction between TM III and TM VII in a G-protein-coupled receptor.

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