TM2-TM7 Interaction in Coupling Movement of Transmembrane Helices to Activation of the Angiotensin II Type-1 Receptor

Shin-ichiro Miura, Jingli Zhang, John Boros and Sadashiva S. Karnik*

Department of Molecular Cardiology, Lerner Research Institute, The Cleveland Clinic Foundation,
9500 Euclid Avenue, NB50, Cleveland, Ohio, 44195, USA

Running Title: Activation-induced Conformational Changes of AT1 Receptor

*Corresponding Author:
Sadashiva S. Karnik, Ph.D.
Department of Molecular Cardiology
Lerner Research Institute
Cleveland Clinic Foundation
9500 Euclid Avenue, NB50
Cleveland, Ohio 44195
216-444-1269 / Fax 216-444-9263
Email: karniks@ccf.org
SUMMARY

Agonist-induced rigid body motion of transmembrane (TM-) helices has been established as a unifying mechanism in the activation of the G-protein-coupled receptors. In attempts to measure specific conformational transitions during the activation of the type 1 receptor for angiotensin II (AT₁), we found a decrease in accessibility of Cys⁷⁶ in the second TM helix suggesting that the orientation of TM2 is altered (Miura and Karnik, *J. Biol. Chem.* 277: 24299-24305, 2002). Now we provide evidence that the TM2 helical movement plays a role in regulating the activated-state of the AT₁ receptor and this role may involve an interaction between TM2 and TM7. Alanine substitution of native Cys²⁹⁶ in TM7 leads to increased accessibility of Cys²⁸⁹ and diminished response to bound agonist. Both effects of the C296A mutation are suppressed when combined with F77A and N111G mutants. The TM7 conformation and the sensitivity of Cys²⁸⁹ altered by C296A mutation is suppressed by the F77A mutation in TM2 in order to salvage function. We show that the F77A mutant alters orientation of both TM2 and TM7 but does not induce constitutive activity in suppressing the C296A mutant-effects. Thus, interaction of TM2 and TM7 is important for transmembrane signal transduction in the AT₁ receptor.
INTRODUCTION

Recent advances in the human genome project has yielded an estimate of > 2000 transmembrane (TM) receptors that are members of the G protein-coupled receptor (GPCR) superfamily. Diverse endogenous ligands such as endocrine hormones, neurotransmitters, ions, peptides, proteases, glycoproteins and sensory signals can activate these receptors (1). Activated GPCRs recruit intracellular heterotrimeric G proteins and stimulate GTP/GDP exchange to initiate receptor-specific signals. Although, their structure-function relationships vary markedly, a seven-TM helical structure is essential for signal transduction by GPCRs. Therefore, activation of GPCRs has been proposed to involve a common molecular mechanism i.e., rigid body movement of TM helices (2, 3). Previous studies of prototypical GPCRs demonstrated that activation induces relative movement of TM3, TM6 and TM7 (1–3).

The type 1 (AT₁) receptor for the octapeptide hormone angiotensin II (Ang II) is a member of the GPCR superfamily (Fig. 1). It is an important target for drug development, since abnormalities in its function are linked to hypertension, water-electrolyte imbalance, hyperaldosteronism, cardiac hypertrophy and heart failure (4). Activation of AT₁ receptor by Ang II and its subsequent coupling to G proteins, Gq/11, results in phospholipase C activation, inositol phosphate accumulation and smooth muscle contraction (4). Ang II-binding involves TM3, TM5, TM6 and TM7 helices. Following binding, interaction of Ang II–Tyr⁴ with Asn¹¹¹ and Ang II–Phe⁸ with His²⁵⁶ (see Fig. 1) leads the AT₁ receptor from an inactive (R) to activated state (R*) (5, 6). Substitution of Asn¹¹¹ in the TM3 results in constitutive activation of the receptor (5). The conservation of agonist-receptor contacts and induction of constitutive activation indicates that Ang II may activate AT₁ receptor by inducing rigid body motion of TM-helices, as in the case of other GPCRs. However, the specific conformational changes essential for activation of the AT₁ receptor are mostly unknown.

Recently, we showed that reduction of Asn¹¹¹ side chain size in the AT₁ receptor resulted in different degrees of constitutive activation (7). Reporter Cys-accessibility mapping (RCAM) in these mutants demonstrated different degrees of accessibility of the TM2 residue, Cys⁷⁶, to methanethiosulfonyl-
ethylaminoacetate (MTSEA) derivatization. This provided the first evidence of TM2 helical movement during AT₁ receptor activation. An exhaustive mapping of conformational changes in other helices was, however, hampered because the MTSEA reactivity of the remaining native Cys residues is not known. Therefore, we reasoned that the construction of an AT₁ receptor CYS⁻ mutant that lacks all free Cys residues would be a useful template for mapping the conformational changes in all TM helices. Hence, we constructed and evaluated single or combinatorial replacements of free Cys residues in the AT₁ receptor. We unexpectedly discovered that substitution of native Cys²⁹⁶ induces a change in the orientation of TM7, accompanied with functional defects. We show here that both effects induced by C296A mutation are abolished in a mutant located in the TM2. Taken together, these findings expose a critical role of TM2-TM7 interaction in coupling helical movements to G protein-activation.
EXPERIMENTAL PROCEDURES

Materials—The highly reactive, sulfhydryl-specific alkylating reagents used were CH₃SO₂-SCH₂CH₂NH₃⁺ (methanethiosulfonyl ethyl-ammonium [MTSEA⁺], adduct size about 4.726Å), CH₃SO₂-SCH₂CH₂NMe₃⁺ (methanethiosulfonyltrimethylammonium [MTSET⁺], adduct size about 6.058Å), CH₃SO₂-SCH₂CH₂SO₃⁻ (methanethiosulfonylethyl-sulfonate [MTSES⁻]) and CH₃SO₂-SCH (methanethiosulfonyl-methyl), which were purchased from Toronto Research Chemicals, Inc., Ontario, Canada. [Sar¹,Ile⁸]Ang II and [Sar¹]Ang II were purchased from Bachem, Torrance, CA. ¹²⁵I–[Sar¹,Ile⁸]Ang II, (Sp. Activity 2200 Ci/mmol) was purchased from Dr. Robert Speth, Washington State University, Pulman, WA. Losartan was a gift from DuPont Merck Co., Wilmington, DE.

Mutagenesis and Expression of the AT₁ Receptor and Membrane Preparation—The synthetic rat AT₁ receptor gene, cloned in the shuttle expression vector pMT-2, was used for expression and mutagenesis, as described in our earlier studies (7). To express the AT₁ receptor protein, 10 µg of purified plasmid DNA per 10⁷ cells was used in transfection. COS1 cells (American type culture collection, Rockville, MD), cultured in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), were transfected by the DEAE-dextran method. Transfected cells cultured for 72 h were harvested and cell membranes were prepared by the nitrogen Parr bomb disruption method in the presence of protease inhibitors. The final membrane suspension was at 1 mg/ml protein. The receptor expression was assessed in each case by immunoblot analysis (not shown) and by ¹²⁵I–[Sar¹,Ile⁸]Ang II saturation binding analysis.

Radioligand Binding Studies—¹²⁵I–[Sar¹,Ile⁸]Ang II-binding experiments were carried out under equilibrium conditions, as previously described (7). Membranes expressing the wild-type (WT) or the mutant receptor were incubated with 300 pM ¹²⁵I–[Sar¹,Ile⁸]Ang II for 1 h at 22 °C in a 250 µL volume. Nonspecific binding of the radioligand was measured in the presence of 1 mM ¹²⁷I–[Sar¹,Ile⁸]Ang II. The binding experiments were stopped by filtering the binding mixture through Whatman GF/C glass fiber filters, which were extensively washed further with binding buffer. The bound ligand fraction was determined from the counts per minute (CPM) remaining on the membrane. Binding kinetics were
determined using the computer program Ligand®. The K_d and B_max values represent the mean ± S.E.M. of three to five independent determinations.

Reactions with MTS Reagents on Binding—Aliquots of cell membranes (20 µl) were incubated with or without MTS-reagents at the stated concentrations (0.1–12.5 mM) at 22 °C for the indicated time (2–10 min) in 20 mM HEPES buffer (pH 7.4). The reaction mix was then diluted 100-fold with cold buffer to stop the reaction and centrifuged for 10 min at 16,000 × g at 4 °C, then resuspended in 200 µl. A 150-µl aliquot was used for 125I-[Sar^1,Ile^8]Ang II binding analysis. The percent inhibition of 125I-[Sar^1,Ile^8]Ang II binding was calculated using the formula 1—[(specific binding after MTS-reagent) / (specific binding without reagent)] × 100%. The values represent the mean ± S.E.M. of four to 10 independent determinations.

Inositol Phosphate Formation Studies—Semi-confluent COS1 cells transfected in 60-mm petri dishes were labeled for 24 h with [3H]myoinositol (1.5 µCi/ml), specific activity 22 µCi/mol (Amersham), at 37 °C in DMEM containing 10% FBS. The labeled cells were washed three times with Hank’s balanced salt solution (HBSS) and incubated with HBSS containing 10 mM LiCl for 20 min. Agonists were then added and incubation continued for another 45 min at 37 °C. At the end of incubation, the medium was removed, and the total soluble IP was extracted from the cells by the perchloric acid extraction method, as described previously (7). The values represent the mean ± S.E.M. of three to four independent determinations.
RESULTS AND DISCUSSION

C296A mutation Alters Accessibility of Native Cystein(s) to MTSEA—Exposure of native Cys residues to water-accessible ligand pocket was measured by employing a highly reactive, sulfhydryl-specific reaction with MTSEA. Water-exposed Cys residues ionize and react a billion times faster with MTSEA which is >3000-fold more soluble in water than membrane (7–10). This reaction results in the addition of a positively charged -sulfonylmethylammonium group onto water exposed Cys via a mixed disulfide bond. When the Cys is in the ligand pocket, the modification interferes with the ^125^I-[Sar^1^, Ile^8^]Ang II binding either through steric hindrance or electrostatic repulsion.

Exposure of the wild-type AT₃ receptor to MTSEA⁺ reagent for ≈10 min abolished specific binding of the peptide antagonist ^125^I-[Sar^1^, Ile^8^]Ang II by nearly 70% (Fig. 2 A). The kinetics of inhibition is dependent on the concentration of the MTS-reagent and the time of reaction. Based on the observation presented in Figure 2A, a 2 min reaction with 2.5 mM MTSEA⁺ was used for the analysis of various mutants. Binding inhibition caused by MTSEA-reaction is irreversible and leads to a decrease in the Bₘₐₓ (Fig. 2A). We reported earlier that the negatively charged MTSES⁻ and the uncharged methyl-MTS did not inhibit ^125^I-[Sar^1^, Ile^8^]Ang II binding because these two reagents reacted poorly with the wild-type AT₃ receptor. In the presence of ^125^I-[Sar^1^,Ile^8^]Ang II, the AT₃ receptor was protected against reaction with MTSEA⁺ (see Ref. 7).

To identify one or more native Cys residue(s) of the AT₃ receptor that reacted with MTS-reagents to inhibit antagonist binding we replaced individual Cys residues with Ala (Fig. 1). These residues located in the putative TM (Cys⁷⁶, Cys¹²¹, Cys¹⁴⁹, Cys²⁸⁹ and Cys²⁹⁶) and cytoplasmic (Cys³⁵⁵) domains are thought to be not involved in disulfide bond formation. A CYS⁻ mutant AT₃ receptor which lacked all non-disulfide bonded Cys residues was constructed. Three double Cys replacement mutants and three triple Cys mutants also resulted during the stepwise construction of CYS⁻ mutant AT₃ receptor (see Table 1). The affinity of these mutants was not significantly different from that of the WT receptor for ^125^I-[Sar^1^,Ile^8^]Ang II (Table I), indicating that single or multiple Ala substitutions did not affect the global conformation of the receptor. We conclude that preservation of the disulfide bonds proposed for the AT₃
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Receptor in previous reports (4) is necessary and sufficient for generating a functional receptor (Fig. 1) and the other native Cys residues are not likely to be involved in conferring ligand selectivity. This finding also indicates that native Cys residues in the AT1 receptor could be used as conformational sensors.

Reaction with 2.5 mM MTSEA+ for 2 min inhibited antagonist binding by ≈ 28 ± 4 % in the WT, C121A, C149A and C355A mutant receptors. In the C76A and CYS− mutants, binding was resistant to reaction with MTSEA+ (Fig. 2A & B). These findings are consistent with our earlier conclusion that inhibition of 125I-[Sar1,Ile8]Ang II binding is due to reaction of MTSEA+ with Cys76 in the TM2 helix and the remaining free Cys residues are not involved (7). The C289A mutant consistently displayed reduced sensitivity to MTSEA, but the magnitude of its reactivity is very small. In contrast, both the magnitude and rate of initial reaction with the MTSEA+ was enhanced in the C296A mutant receptor suggesting that this mutation may have perturbed the conformation of the receptor (Fig. 2A & B). Competition binding studies with the AT1 receptor-selective nonpeptide antagonist losartan revealed a 11-fold reduction of affinity for C296A compared to the WT receptor. Substitution of Cys296 with Gly, Ile and Phe also reduced the affinity for losartan (Table1). These mutants also displayed reduced coupling to IP production. Previous mutagenesis studies have indicated that TM7 plays an important role in nonpeptide antagonist-selectivity and signal activation (6). Based on these observations, we speculate that C296A mutation perturbs receptor conformation leading to increased accessibility of one or more native Cys residues.

Reaction of the TM7 Residue, Cys289, with MTSEA is Enhanced in the C296A Mutant—The accessibility of remaining native Cys residues in various combinations with the C296A mutation was investigated. As illustrated in Fig. 2C, the C296A mutant contains at least two MTSEA-reactive cysteines. The binding of 125I-[Sar1,Ile8]Ang II is not sensitive to MTSEA-reaction when both Cys76 and Cys289 are replaced in the C296A mutant. Only 50% of MTSEA-inhibition of binding was observed in C76A/C296A/C355A, C76A/C149A/C296A, C76A/C121A/C296A and C76A/C296A mutants compared to the C296A mutant. The reduction is consistent with loss of Cys76 residue which is normally reactive. The MTSEA-sensitivity of the mutant C289A/C296A compared with that of the mutants
C76A/C289A/C296A, C76A, C289A and C296A indicates that the remaining 50% inhibition is consistent with acquired reactivity of Cys\textsuperscript{289} in these mutants (Fig. 2C). The analysis indicates that the C296A mutation induced an increase in the accessibility of Cys\textsuperscript{289} without altering the accessibility of Cys\textsuperscript{76}.

The structural consequence of C296A mutation was simulated in a homology model of the AT\textsubscript{1} receptor molecule (D. C. Teller, V.C. Yee and S. Karnik, unpublished) constructed based on X-ray diffraction coordinates of bovine rhodopsin (1, 12). In our model Cys\textsuperscript{296} side-chain is located in an elongated part of the helix that bends toward TM2, and its sidechain is buried. Conformation simulated with Ala\textsuperscript{296} indicated more flexibility to the TM7-region that would allow interaction with the surrounding lipids. The C296A mutation potentially could cause an increased sensitivity of Cys\textsuperscript{289} through local change of TM7-conformation without affecting other native Cys residues in the AT\textsubscript{1} receptor. We conclude that the acquired MTSEA-sensitivity of Cys\textsuperscript{289} due to C296A mutation reflects a conformational change of TM7 (rotating and/or tilting) resulting in movement of Cys\textsuperscript{289} into water-accessible ligand binding pocket.

The C296A-induced Accessibility of Cys\textsuperscript{289} is Masked by the F77A Mutation in TM2—
Pharmacological evidence for a specific interaction between TM2 and TM7 in the AT\textsubscript{1} receptor was reported in an earlier study (11). Consistent with this suggestion, the AT\textsubscript{1} receptor molecular-model also indicated that the TM7 region containing the Cys\textsuperscript{296} interacts extensively with TM2. Therefore, we looked for compensatory mutations in the TM2 helix and found that Ile, Cys Ala and Gly substitutions of Phe\textsuperscript{77} in TM2 rescued the C296A-induced MTSEA-sensitivity of Cys\textsuperscript{289} (see Fig. 2D). These substitutions produced a gradual decrease of MTSEA-inactivation of binding. Cys\textsuperscript{76} is not sensitive to MTSEA-inactivation in these mutants as it is in the wild-type. This conclusion is supported by the MTSEA-resistance of the double mutants, F77A/C76A and F77A/C289A. We speculate that smaller side chains substituted for Phe\textsuperscript{77} perhaps alter the environment of Cys\textsuperscript{76}, a residue that is normally MTSEA-accessible.

In the AT\textsubscript{1} receptor molecular model, the side chain of Phe\textsuperscript{77} is located at the boundary between the water-accessible ligand pocket and hydrophobic protein core with bonding proximity to residues Tyr\textsuperscript{292}-
Phe$^{293}$-Asn$^{294}$ located on TM7. Therefore, smaller residues substituted for Phe$^{77}$ could potentially cause a rearrangement of both TM2 and TM7 within the TM bundle to accommodate the space created in the protein core. Consequently, the environment around the conformational sensor Cys$^{76}$ on TM2, could be more hydrophobic. Cysteines placed in hydrophobic environment do not ionize, hence, MTSEA-insensitivity of Cys$^{76}$ in the F77A mutants is consistent with the model. Whether the small residues substituted for Phe$^{77}$ affect TM7 conformation is not clear. The MTSEA resistance noted in the F77A mutant does not necessarily exclude a potential conformational change in TM7. Since the conformation-sensor residue, Cys$^{289}$, in TM7 is not very reactive (see Fig. 1A), a further decrease in the MTSEA-sensitivity, if it occurred in the Phe$^{77}$ mutants, is difficult to detect. An increase in its reactivity should be measurable, but not actually found in these mutants.

The F77A/C296A double mutant displayed MTSEA-inactivation kinetics which is comparable to that of the WT receptor. The MTSEA-sensitivity of Cys$^{289}$ induced by the C296A mutation and the accessibility of Cys$^{76}$ decreased by the F77A mutation are masked in this double mutant. Rather surprisingly, the MTSEA-inactivation of F77A/C289A/C296A mutant demonstrated that ≈75% of inhibition of $^{125}$I-[Sar$^{1}$,Ile$^{8}$]Ang II binding is due to reaction with Cys$^{76}$. The inactivation of the triple mutant, F77A/C76A/C296A indicated that ≈ 25% of inhibition is due to accessibility of Cys$^{289}$. Furthermore, the change in MTSEA-reactivity was accompanied by an increase of affinity for losartan and restoration of agonist-induced IP stimulation in comparison to the C296A mutant (Table 1). Reactivity of other native Cys residues did not change (Fig. 2D). Thus, the properties of C77A/C296A mutant are more “wild-type-like” than “F77A-like” or “C296A-like”.

The local effects caused by F77A and C296A individual mutations respectively on TM2 and TM7 could cancel out in the F77A/C296A double mutant. The conformation of the TM helical bundle in this mutant mimics the packing of helices in the WT receptor as suggested by antagonist binding profiles, regained MTSEA-sensitivity of Cys$^{76}$ and the decreased sensitivity of Cys$^{289}$. In rhodopsin, interaction between TM3 and TM6 was initially deduced from compensatory effects of individual mutations and was later confirmed by the high-resolution structure (2, 3). Similarly, an ionic lock between cytoplasmic ends of TM3 and TM6 was demonstrated in β2-adrenergic receptor (2, 10). Functional interaction between
TM2 and TM7 is observed in many GPCRs. Spatial proximity and intermolecular interactions between TM2 and TM7 have been observed in the crystallographic structure of bovine rhodopsin (1, 12).

**Distinct Mutation-induced Helical Motions Affect Receptor-activation Differently**—The decreased reactivity of Cys\(^{76}\) is a prominent signature of activated receptor conformation in AT\(_1\) receptor (7). Hence, the F77A-induced decrease of Cys\(^{76}\)-accessibility might indicate altered function. To evaluate the functional consequences of the putative motion of helices observed in Phe\(^{77}\) and Cys\(^{296}\) mutants, we measured stimulation of G\(_q\)-PLC-mediated IP production in COS1 cells expressing the WT, F77A, C296A and F77A/C296A receptors. To account for variation of dose-response relationships due to variable receptor expression, IP production per pmol receptor was estimated in each case (see Fig. 3).

The [Sar\(^1\)]Ang II potency (EC\(_{50} 6.2 \pm 2\) nM) was not significantly different between the WT and F77A/C296A mutant. Compared to these two receptors, the F77A mutant displayed 3 to 5-fold increase of potency (EC\(_{50} 2.0 \pm 1\) nM) and significantly higher maximal IP response. Previously, a F77S mutant with similar properties was reported (13). To evaluate whether this potency change is due to possible constitutive activation, the basal activities were measured at different receptor densities. The slope of receptor-density to basal-activity relationship for the WT (0.08 ± 0.001) was not significantly different from that for the F77A mutant (0.09 ± 0.004). Thus, the F77A mutation increased the ligand potency but does not alleviate “the constraint” to induce constitutive activity. In contrast, in the C296A mutant both the potency of [Sar\(^1\)]Ang II (EC\(_{50} 14.1 \pm 4\) nM) and maximal response are reduced, indicating that the C296A mutation-induced effect is unfavorable to receptor activation. This finding implies that the C296A-induced TM7 motion and acquired MTSEA-sensitivity of Cys\(^{289}\) is perhaps a signature for defective receptor activation.

We next evaluated these mutation-induced effects on the constitutive activity of the N111G receptor. Constitutive activation results from loss of constraint i.e. a lowering of thermodynamic barrier for conformational changes essential for receptor-activation (5). Figure 3 illustrates elevation of agonist-independent basal intracellular IP accumulation in the N111G-transfected COS1 cells, as reported earlier from this lab. The slope of this basal activity was (1.21 ± 0.2) not significantly affected by introduction of F77A (slope, 0.98 ± 0.09) and F77A/C296A (slope, 0.81 ± 0.07) mutations. The constitutive activity
of N111G receptor was not abolished by introduction of C296A mutation; although, significant ($P > 0.001$) reduction of the basal activity was observed in the N111G/C296A double mutant (slope, $0.98 \pm 0.2$). The [Sar$^1$]Ang II-dependent maximal response was significantly ($P < 0.05$) higher in the F77A/N111G mutant but was not substantially different in the remaining three mutants (see Fig. 3).

**TM2-TM7 Interaction in AT$_1$ Receptor Function**—The F77A mutation-induced conformation favors ligation of the receptor with the agonist, observed as increased potency of [Sar$^1$]Ang II. However, the lack of synergistic effect on the constitutive activity of N111G mutant indicates that Phe$^{77}$ does not participate in the early step(s) of receptor activation. Most likely, the F77A mutation prolongs the agonist activation, accounting for the higher agonist-dependent maximal response observed in both WT and the N111G mutant. We propose that Phe$^{77}$ regulates maximal response of the agonist-bound receptor and this role may depend critically on the ability of TM2 to dynamically interact with TM7. The observations reported here indicate that distinct TM2 and TM7 motions are a mark of functional consequence, thus implicating mutation-induced helical motion in function. For instance, a decrease of Cys$^{76}$-accessibility indicates TM2 motion in favor of activated receptor conformation, whereas an increase of Cys$^{76}$-accessibility indicates basal receptor conformation. One of our goals is to decipher unique conformational signatures for each helix and inter-helical loop in the AT$_1$ receptor, to enable prediction of functional states of the receptor in a cell free system.

Our finding here suggests that a mechanism for dynamic TM2–TM7 interaction is conceivably involved in coupling agonist binding to receptor activation. Interaction between Asp$^{74}$ in TM2 and Tyr$^{292}$ in TM7 in the AT$_1$ receptor was predicted. On the basis of this interaction it was proposed that TM2 and TM7 helices move closer to each other in the activated state (11). The Asp$^{74}$—Tyr$^{292}$ interaction proposed in the earlier models was not found in our homology model of the AT$_1$ receptor. The model also rules out a direct interaction between Phe$^{77}$ and Cys$^{296}$, the two residues studied in this report. However, the basal structure predicted in our modeling simulation lays the ground work for mechanical interaction between these helices. The TM2–TM7 interface in the model involves bulky aromatic residues (Phe$^{77}$, Tyr$^{292}$ and Phe$^{293}$) mixed with strong polar side chains, forming an extensive hydrogen bonding network \{Asn$^{69}$—Tyr$^{302}$, Asp$^{74}$—back bone carbonyl between Asn$^{294}$ and Asn$^{295}$, Asp$^{74}$—water—Asn$^{298}$, and
Thr\textsuperscript{80}—Tyr\textsuperscript{292} between TM2 and TM7. Previous mutagenesis studies targeting Asp\textsuperscript{74}, Phe\textsuperscript{77}, Tyr\textsuperscript{292}, Asn\textsuperscript{294}, Asn\textsuperscript{295}, Asn\textsuperscript{298}, and Tyr\textsuperscript{302} indicate that these residues either affect agonist-dependent activation or cause structural defects affect the affinity for ligands (6, 11, 13). Photo-cross linking studies identified the Phe\textsuperscript{293}—Asn\textsuperscript{294} dipeptide region of TM7 as a potential host site for the interaction of Phe\textsuperscript{8} side chain of Ang II (15). Therefore, we propose that any change, brought about by agonists, to the hydrophobic and hydrogen bonding interactions stabilizing TM2-TM7, are likely to induce motion of these helices. It appears to us that relative movement of both TM2 and TM7 is critically important for regulation of agonist-activated receptor function.

Transmembrane Helical Movements in GPCR Activation—Because a large number of GPCRs are capable of promoting signal transduction in response to diverse ligands, ligand-receptor interactions have been studied in great detail. The mechanism by which the binding of ligand leads to activation of the receptor is only recently beginning to be unraveled (2, 3). These studies carried out in prototypical GPCRs, rhodopsin, \( \beta_{2} \)-adrenergic receptor, dopamine receptor, \( \alpha_{-} \)-adrenergic receptors and muscarinic receptors suggested that receptor-activation involves critical conformational changes in TM3 and TM6 (2, 3). In both rhodopsin and \( \beta_{2} \)-adrenergic receptor, spectroscopic studies have provided evidence for a rigid body motion of the TM-helices. Preventing TM3–TM6 motion prevents receptor dependent G-protein activation, implying that movement of these TM-helices is critical for activation. Although activation generally requires initial binding of the ligand, mutation induced receptor activation can be observed in many GPCRs. This mode of activation also involves movement of TM3 and TM6 suggesting that TM-helical movements may be similar among members of GPCR family. Smaller movements in the loop connecting TM1 to TM2, and in TM7, have been mapped in rhodopsin. By constructing an activating metal-ion site between TM3 and TM7, motion of these two helices is confirmed in the \( \beta_{2} \)-adrenergic receptor. Only minor changes have been reported for TM4 and TM5. The established motion of TM3 and TM6 in more than three GPCRs, however, does not exclude that movement of other domains may not contribute to receptor activation. In recent fluorescence spectroscopic studies of \( \beta_{2} \)-adrenergic receptor, evidence for multiple sequential conformational changes was obtained (16), which is reminiscent of multiple intermediates involved in the photoactivation of rhodopsin. Sequential
conformational changes in the activation process of GPCRs may involve general and specific movements of TM-helices to distinct positions with characteristic time constraints (see Refs. 2 and 3 for detailed discussion).

Given the fact that residues corresponding to Asp\textsuperscript{74} in TM2 and the \textsuperscript{298}-NPLFY-\textsuperscript{302} in TM7 of the AT\textsubscript{1} receptor are highly conserved in the GPCR superfamily, the functional significance of TM2–TM7 interactions uncovered in this study may be common to many GPCRs. Interaction of TM2–TM7 has been postulated in the \(\alpha\)-adrenergic receptor, gonadotrophin releasing hormone receptor, vasopressin receptor, oxytocin receptor and endothelin receptor (11). Whether the effects we report here on stabilization of agonist-bound receptor and prolongation of active state is a common phenotype in diverse GPCRs requires further study.
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REFERENCES


FOOTNOTES

1Abbreviations: Ang II, angiotensin II [Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-COO⁻]; AT₁, angiotensin II type-1; RCAM, reporter cysteine accessibility mapping; MTSEA, methanethiosulfonylethyl-ammonium; MTSET, methanethiosulfonylethyl-trimethyl-ammonium; TM, transmembrane; IP, inositol phosphate; SH, sulfhydryl group; R, the basal inactive state; R*, activated state; WT, wild-type
FIGURE LEGENDS

Fig. 1. A secondary structure model of rat AT_1 receptor revised based on the structure of bovine rhodopsin (12). Native Cys residues in the receptor are highlighted and residues indicated by ♦ are the focus of the present study. Extracellular Cys^{18}–Cys^{274} and Cys^{101}–Cys^{180} form disulfide bonds, the cytoplasmic Cys^{355} may be palmitoylated, and five Cys residues in the TM domain are thought to be free thiols.

Fig. 2. (A) Inhibition of \(^{125}\text{I}\)-[Sar\(^1\), Ile\(^8\)]Ang II specific binding after treatment with 2.5 mM MTSEA\(^+\). After the MTSEA\(^+\) reaction with COS cell membrane for the indicated time, the reaction mixture was diluted 100-fold, and membranes were centrifuged and suspended to carry out saturation binding analysis as described in methods. Maximal specific binding to each control sample (adjusted to ~30,000 CPM) without MTSEA\(^+\) reagent is represented as 100%. (B–D) Inhibition of specific binding to the WT and mutant receptors, after 2-min reactions with 2.5 mM MTSEA\(^+\). Values of five independent experiments (mean ± S.E.M.) are shown. Analysis of variance showed significant differences (\(P < 0.05\)) indicated between samples compared, * and †, in the reaction with MTSEA\(^+\). The differences between the means were evaluated by the most significant difference procedure.

Fig. 3. IP production in response to varying the [Sar\(^1\)]Ang II concentration in COS1 cells transfected with WT and specified mutants of the AT1 receptor. The values plotted are from three independent transfection experiments (mean ± S.E.M.).
TABLE 1

Ligand-affinity, $B_{\text{max}}$ and maximal and IP stimulation values for wild-type and mutant AT1 receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_d$ (nM)</th>
<th>$\text{[Sar}^1,\text{Ile}^8]\text{Ang II}$ (nM)</th>
<th>Losartan (nM)</th>
<th>$B_{\text{max}}$ (pmol/mg)</th>
<th>Basal</th>
<th>$\text{[Sar}^1]\text{Ang II}$-stimulated</th>
<th>* IP production [% of WT]</th>
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<tr>
<td>WT</td>
<td>0.37 ± 0.07</td>
<td>12 ± 2</td>
<td>4.5 ± 0.3</td>
<td>6 ± 3</td>
<td>100 ± 5</td>
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<tr>
<td>CYS−</td>
<td>1.07 ± 0.08</td>
<td>26 ± 4</td>
<td>5.1 ± 0.2</td>
<td>8 ± 4</td>
<td>88 ± 10</td>
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<tr>
<td>C76A</td>
<td>0.87 ± 0.11</td>
<td>7.3 ± 2</td>
<td>3.9 ± 0.5</td>
<td>6 ± 3</td>
<td>94 ± 8</td>
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<tr>
<td>C121A</td>
<td>0.47 ± 0.14</td>
<td>47 ± 10</td>
<td>2.9 ± 0.5</td>
<td>3 ± 1</td>
<td>96 ± 4</td>
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<tr>
<td>C149A</td>
<td>1.27 ± 0.24</td>
<td>14 ± 3</td>
<td>4.5 ± 0.6</td>
<td>3 ± 1</td>
<td>97 ± 10</td>
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<tr>
<td>C289A</td>
<td>0.39 ± 0.02</td>
<td>25 ± 4</td>
<td>5.6 ± 0.4</td>
<td>7 ± 3</td>
<td>96 ± 7</td>
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<tr>
<td>C296A</td>
<td>0.37 ± 0.02</td>
<td>132 ± 4</td>
<td>4.8 ± 0.9</td>
<td>4 ± 2</td>
<td>72 ± 4</td>
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<tr>
<td>C355A</td>
<td>0.50 ± 0.04</td>
<td>17 ± 5</td>
<td>3.5 ± 0.3</td>
<td>5 ± 2</td>
<td>91 ± 6</td>
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<tr>
<td>C76A, C296A</td>
<td>0.58 ± 0.04</td>
<td>150 ± 5</td>
<td>4.9 ± 0.2</td>
<td>3 ± 1</td>
<td>69 ± 4</td>
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</tr>
<tr>
<td>C76A, 289A</td>
<td>0.36 ± 0.02</td>
<td>13 ± 1</td>
<td>3.8 ± 0.1</td>
<td>8 ± 2</td>
<td>94 ± 4</td>
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<td></td>
</tr>
<tr>
<td>C76A, C289A, C296A</td>
<td>0.41 ± 0.03</td>
<td>146 ± 7</td>
<td>3.1 ± 0.2</td>
<td>3 ± 1</td>
<td>69 ± 10</td>
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<tr>
<td>F77I</td>
<td>0.30 ± 0.10</td>
<td>22 ± 4</td>
<td>4.2 ± 0.05</td>
<td>8 ± 3</td>
<td>104 ± 10</td>
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<tr>
<td>F77A</td>
<td>0.30 ± 0.09</td>
<td>140 ± 9</td>
<td>5.5 ± 0.4</td>
<td>9 ± 3</td>
<td>115 ± 10</td>
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<tr>
<td>F77C</td>
<td>0.64 ± 0.25</td>
<td>147 ± 2</td>
<td>4.5 ± 0.3</td>
<td>6 ± 3</td>
<td>111 ± 9</td>
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<tr>
<td>F77G</td>
<td>0.34 ± 0.02</td>
<td>145 ± 2</td>
<td>5.0 ± 0.4</td>
<td>9 ± 4</td>
<td>110 ± 10</td>
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<tr>
<td>C296G</td>
<td>0.68 ± 0.08</td>
<td>112 ± 3</td>
<td>3.2 ± 0.3</td>
<td>3 ± 1</td>
<td>54 ± 6</td>
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<tr>
<td>C296I</td>
<td>0.54 ± 0.08</td>
<td>114 ± 1</td>
<td>4.7 ± 0.3</td>
<td>1 ± 2</td>
<td>75 ± 7</td>
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<tr>
<td>C296F</td>
<td>0.48 ± 0.05</td>
<td>158 ± 6</td>
<td>0.9 ± 0.2</td>
<td>5 ± 2</td>
<td>41 ± 3</td>
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<tr>
<td>C76A/F77A</td>
<td>1.35 ± 0.23</td>
<td>64 ± 10</td>
<td>5.6 ± 0.1</td>
<td>4 ± 1</td>
<td>99 ± 4</td>
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<tr>
<td>F77A,C289A</td>
<td>0.41 ± 0.04</td>
<td>88 ± 9</td>
<td>4.5 ± 0.3</td>
<td>8 ± 2</td>
<td>93 ± 8</td>
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</table>
### Activation-induced Conformational Changes of AT$_1$ Receptor

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Specific Binding</th>
<th>Nonspecific Binding</th>
<th>Total Specific Binding</th>
<th>Nonspecific Binding</th>
</tr>
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<tbody>
<tr>
<td>F77A, C296A</td>
<td>0.52 ± 0.02</td>
<td>21 ± 2</td>
<td>4.4 ± 0.2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>F77A, C298A,C296A</td>
<td>0.48 ± 0.09</td>
<td>23 ± 3</td>
<td>4.3 ± 0.3</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>C76A, F77A, C298A</td>
<td>0.39 ± 0.04</td>
<td>69 ± 7</td>
<td>4.1 ± 0.4</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>C76A, F77A, C296A</td>
<td>0.74 ± 0.02</td>
<td>23 ± 3</td>
<td>4.9 ± 0.1</td>
<td>6 ± 2</td>
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<tr>
<td>N111G</td>
<td>0.36 ± 0.02</td>
<td>230 ± 30</td>
<td>3.6 ± 0.1</td>
<td>42 ± 2</td>
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<tr>
<td>F77A,N111G</td>
<td>0.17 ± 0.02</td>
<td>410 ± 30</td>
<td>5.2 ± 0.2</td>
<td>65 ± 8</td>
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<tr>
<td>N111G, C296A</td>
<td>0.32 ± 0.04</td>
<td>155 ± 20</td>
<td>4.4 ± 0.5</td>
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<td>F77A, N111G, C296A</td>
<td>0.38 ± 0.13</td>
<td>65 ± 9</td>
<td>4.7 ± 0.1</td>
<td>47 ± 5</td>
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</tbody>
</table>

The values shown are means and standard errors for 4–10 independent determinations for each mutant. In all experiments an excess of $^{125}$I(Sar$^1$,Ile$^8$)Ang II was used to obtain > 99% receptor in the ligand-bound form. The total specific binding was adjusted to be within 10% of total CPM added to each sample, and the nonspecific binding was < 5% of specific binding.

*The values represent total inositol phosphates measured in COS1 cells transfected with WT and mutant AT$_1$ receptor expression plasmids (10 µgDNA/10$^7$ cells) in three independent experiments. IP value measured in COS1 cells transfected with expression plasmid without the AT$_1$ receptor gene (< 0.2% compared to activated WT) was subtracted from all values. The average of agonist-stimulated (by 10$^{-6}$M [Sar$^1$]Ang II) IP values for the WT. AT$_1$ receptor was 10,000 ± 400 CPM/pmol receptor. This value was taken as 100% to represent the effect of various different substitutions on the function of AT$_1$ receptor. The expression level of receptor estimated for different mutants varied within 2-fold.
Figure 1
Figure 2

A

Wild-type

B

% Inhibition of 125I-[Sar1,Ile8]Ang II

* p < 0.05 vs. wild-type
† p < 0.05 vs. wild-type

C

% Inhibition of 125I-[Sar1,Ile8]Ang II

* p < 0.05 vs. wild-type
† p < 0.05 vs. wild-type

D

% Inhibition of 125I-[Sar1,Ile8]Ang II

* p < 0.05 vs. wild-type
† p < 0.05 vs. F77A
Figure 3
TM2-TM7 interaction in coupling movement of transmembrane helices to activation of the angiotensin II type-1 receptor
Shin-ichiro Miura, Jingli Zhang, John Boros and Sadashiva S. Karnik

J. Biol. Chem. published online November 21, 2002

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