Constitutive Activation of the Angiotensin II Type 1 Receptor Alters the Spatial Proximity of Transmembrane 7 to the Ligand-binding Pocket*

Antony A. Boucard¶, Marise Roy, Marie-Ève Beaulieu, Pierre Lavigne, Emanuel Escher§, Gaëtan Guillemette, and Richard Leduc¶

From the Department of Pharmacology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

G protein-coupled receptors (GPCRs)1 comprise a large family of cell surface receptors that mediate diverse responses to a large variety of sensory and hormonal signals. As a common characteristic, they all possess seven membrane-spanning domains that constitute structural support for signal transduction. However, despite a low sequence identity, GPCRs are thought to mediate signal transduction by a mechanism involving common structural movements implicating transmembrane domains (TM) (1). This receptor conformational change is suspected to sustain GTP/GDP exchange on specific guanine nucleotide binding proteins (G proteins) leading to activation of intracellular signaling cascades. Indeed, rotation described by rigid body movement of TM helices as well as translation have been described for activation of rhodopsin and other GPCRs (2–4).

Rhodopsin belongs to class A within the GPCR superfamily. The recent elucidation of the three-dimensional structure of rhodopsin has shed much light into how photosomerization is coupled to conformational change, which leads to G protein signalization (5, 6). Also belonging to class A, the AT1 receptor binds the octapeptide hormone angiotensin II (AngII) and activates the G protein Gq11. This leads to the increase of intracellular Ca2+ levels following hydrolysis by phospholipase C of membranous phosphatidylinositol-bis-phosphate into diacylglycerol and inositol 1,4,5-trisphosphate. Most of the physiological actions of AngII on cardiovascular, endocrine, and neuronal systems are mediated by the AT1 receptor (7). However, the molecular mechanisms by which the AT1 receptor activates those pathways remain elusive. It has been proposed that TM3, TM5, TM6, and TM7 might participate in this activation process by providing a network of interactions through the AngII-binding pocket (8). The dynamics of this network is suspected to be modified following agonist binding, thereby forcing the receptor to form new interactions between TMs. Another way in which an alteration in interactions of TM residues can yield interesting phenotypes is the existence of constitutively active receptors. For instance, it is well documented that N111G mutants within TM3 of the AT1 receptor confer a high level of constitutive activity (9), higher than N111A mutants. This mutation may release receptor constraints between TMs, thereby breaking the existing network involving multiple intramolecular interactions. Indeed, a rigid body movement of TM2 has been described recently for the AT1 receptor following such mutations at Asn111 (10). However, because TM7 bears multiple activation determinants and motifs, such as the 298NXX1302 motif (11), which has been implicated in receptor-mediated G protein activation, their role in the N111G receptor mutant is quite intriguing.

Like in many GPCRs, the binding site of the AT1 receptor is formed among its seven, mostly hydrophobic transmembrane segments and is accessible to charged water-soluble agonists, like AngII. Thus, for this receptor, the binding site is contained in

* This work was supported by a Canadian Institutes of Health Research grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a studentship from the Heart and Stroke Foundation of Canada. This work is completed as part of a Ph.D. thesis.

‡ Recipient of the J. C. Edwards Chair in Cardiovascular Research.

¶ Senior Scholar from the Fonds de la Recherche en Santé du Québec.

To whom correspondence should be addressed: Dept. of Pharmacology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, PQ J1H 5N4, Canada. Tel.: 819-564-5413; Fax: 819-564-5400; E-mail: Richard.Leduc@USherbrooke.ca.

1 The abbreviations used are: GPCR, G protein-coupled receptor; AT1, angiotensin II type 1; AngII, angiotensin II; DMEM, Dulbecco’s modified Eagle’s medium; MTS, methanethiosulfonate; MTSEA, methanethiosulfonylethyl-ammonium; TM, transmembrane domain; ECL, extracellular loop; SCAM, substituted cysteine accessibility method; PBS, phosphate-buffered saline.

Received for publication, June 5, 2003, and in revised form, July 2, 2003

Published, JBC Papers in Press, July 3, 2003, DOI 10.1074/jbc.M305952200
within a water-accessible crevice, the binding pocket, extending from the extracellular surface of the receptor into the transmembrane domain (12). The surface of this crevice is formed by residues that can contact specific determinants on ligands and by other residues that may play a structural role as well as affect binding indirectly. Here, using the substituted cysteine accessibility method (SCAM), we probed the AT1 receptor-binding pocket (13).

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, bacitracin, and soybean trypsin inhibitor were from Sigma. The sulphydryl-specific alkylating reagent used was CH$_2$SO$_2$·CH$_2$NH$_2$. MTSEA, which was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). The cDNA clone of the human AT$_1$ receptor subcloned in the mammalian expression vector pcDNA3 was kindly provided by Dr. Sylvain Meoleu (Université de Montréal). LipofectAMINE and culture media were obtained from Invitrogen. 125I-[Sar$^1$,Ile$^8$]AngII (specific radioactivity, ~2000 Ci/mmol) was prepared with Iodo-GEN (Fierce) according to the method of Fraker and Speck (14) and as previously reported (11).

Numbering of Residues in Transmembrane Domains—Residues in the seventh transmembrane domain of the human AT$_1$ receptor are given in Table I and in Fig. 1, and are consistent with the numbering schemes. First, residues are numbered according to their positions in the human AT$_1$ receptor sequence. Second, residues are also indexed relative to the most conserved residue in the TM in which it is located (N1,N2) (15). N1 refers to the TM number, and N2 refers to the position of the residue relative to the most conserved one with numbers decreasing toward the N terminus and increasing toward the C terminus. By definition, the most conserved residue is assigned the position index 50, e.g. Pro$^{209/307,50}$ and therefore Asn$^{209/307,49}$ and Leu$^{209/307,51}$. This indexing simplifies the identification of aligned residues in different GPCRs.

Oligodeoxynucleotide Site-directed Mutagenesis—Site-directed mutagenesis was performed on the wild type AT$_1$ receptor with the overlap PCR method described elsewhere (16). Mutant receptors were subcloned into HindIII-XbaI sites of the mammalian expression vector pcDNA3. Site-directed mutations were then confirmed by manual and automated DNA sequencing.

Cell Culture and Transfections—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM-glutamine and 10% (v/v) fetal bovine serum. The cells were seeded into 100-mm culture dishes at a density of 2 × 10$^6$ cells/dish. When cells reached approximately 70% confluence, they were washed with serum-free DMEM and transfected with 2 μg of plasmid DNA and 15 μl of LipofectAMINE in 8 ml of serum-free DMEM. The cells were incubated for 5 h at 37 °C, and the media were replaced with a complete DMEM containing 10% fetal calf serum. Transfected cells were trypsinized after 24 h, plated into 12-well plates, and grown for an additional 24 h before MTS treatment. Transfected cells in 100-mm culture dishes were grown for 48 h before binding assays.

Binding Experiments—Cell membrane preparation and binding assays were performed as described previously (12). COS-7 cells were grown for 48 h post-transfection in 100-mm culture dishes, washed once with PBS, and subjected to one freeze-thaw cycle. Broken cells were then gently scraped in washing buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl$_2$), centrifuged at 2500 × g for 15 min at 4 °C, and resuspended in binding buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl$_2$, 0.1% (v/v) bovine serum albumin, 0.01% (v/v) bacitracin). Binding experiments were performed as described previously (16). Mutant receptors were transiently expressed in COS-7 cells transiently expressing the AT$_1$ receptor were treated with varying amounts of the sulphydryl-specific alkylating reagent MTSEA. The extent of reaction after a fixed period of time (3 min) with five concentrations of MTSEA (from 0.005 to 6 mM) (all in excess over the reactive sulhydryl) was fit to (1 − plateau) e$^{-k t}$ + plateau, where plateau is the fraction of residual binding at saturating concentrations of MTSEA, k is the second order rate constant (in m$^{-1}$ s$^{-1}$), C is the concentration of MTSEA (mM), and t is the time (180 s). Fitting was achieved using the Enzfit software from BioSoft (Ferguson, MO).

Protection against MTSEA Reaction by [Sar$^1$,Ile$^8$]AngII—Transfected cells plated into 12-well plates were washed once with PBS and incubated in the presence or the absence of 100 mM [Sar$^1$,Ile$^8$]AngII for 1 h at 16 °C to avoid internalization of membrane expressed receptors. The cells were washed to remove excess ligand and then treated with a concentration of MTSEA that was just sufficient to achieve maximal inhibition of binding to each receptor. The cells were washed three times with ice-cold PBS and were submitted to an acidic wash (150 mM NaCl, 50 mM acetic acid, pH 3.0) to remove bound ligand. The cells were then incubated in binding medium (DMEM, 25 mM HEPES, 0.1% bovine serum albumin, pH 7.4) containing 0.05 mM [Sar$^1$,Ile$^8$]AngII for 3 h at 16 °C. After washing with ice-cold PBS, the cells were lysed with 0.1 N NaOH, and the radioactivity was evaluated by γ counting. The percentage of protection was calculated as [(inhibition in the presence of [Sar$^1$,Ile$^8$]AngII)/(inhibition in the absence of ligand)] × 100%.

Molecular Modeling—All of the calculations were performed on a Silicon Graphics Octane2 workstation. Theoretical structure of the hAT1 receptor was generated by homology modeling based on the crystal structure of the bovine rhodopsin (Protein Data Bank code 1F88). A pair-wise sequence alignment between the two primary structures was performed using the program Homology of Insight II (Accelrys Inc., San Diego, CA). After identification of the structurally conserved regions (transmembrane domains), these coordinates were transferred to the sequence of the hAT1 receptor. The structures of the loop regions of AT1 were modeled using the loop generation program of Insight II. The potential energy of the model structure of hAT1 was minimized by using the molecular modeling package of Insight II/Discover with consistent valence forcefield (18). Two disulfide bridges, Cys$^1$-Cys$^{112}$ and Cys$^{189}$-Cys$^{198}$, were used as distance restraints. A distance-dependent dielectric constant of 4 was used with simple harmonic potential for bond length energy. No cross-terms energies were included, and the peptide bonds were forced to planarity.

RESULTS

Assessment of hAT$_1$ Sensitivity to MTSEA Added Extracellularly—To verify the contribution of the 10 endogenous cysteines (Fig. 1) to ligand binding, we initially performed the SCAM approach on the wild type AT$_1$ receptor. Whole cells transiently expressing the AT$_1$ receptor were treated with varying amounts of the sulphydryl-specific alkylating reagent MTSEA. Fig. 2 shows that treatment with various concentrations of MTSEA had very little effect on the binding properties of the AT$_1$ receptor. No more than a 10% reduction in binding was observed, showing the relatively low contribution of the endogenous cysteines of the receptor to the binding pocket.

Alkylation of the AT$_1$ receptor with other charged MTS agents (methylenebisulfonfyltrimethylammonium and methanethiosulfonfylsulfonate) also did not significantly affect ligand binding (data not shown).

Binding Properties of hAT$_1$, Mutant Receptors Bearing Cysteines in TM7—To establish whether residues of TM7 of the AT$_1$ receptor orient themselves into the binding pocket and hence could potentially affect ligand binding, we generated a series of mutants whereby residues Ile$^{209}/$H11001 and Ile$^{307}/$H11002 were replaced by cysteines. Each mutant receptor was transiently expressed in COS-7 cells. To assess the conservation of global conformation of these receptors after such substitutions, pharmacological parameters describing the equilibrium binding of the radiolabeled antagonist 125I-[Sar$^1$,Ile$^8$]AngII such as $K_d$ and $B_{max}$ were determined (Table I). All of the calculated was as $[1−(\text{specific binding after the MTS reagent/specific binding without the reagent})] × 100%$.

Measurement of the Second Order Rate Constant for MTSEA Reaction—The second order rate constant ($k$) for the reaction of MTSEA with each susceptible receptor mutants was estimated by determining the extent of reaction after a fixed period of time (3 min) with five concentrations of MTSEA (typically from 0.005 to 6 mM) (all in excess over the reactive sulhydryl). The fraction of initial binding $Y$ was fit to (1 − plateau) e$^{-k t}$ + plateau, where plateau is the fraction of residual binding at saturating concentrations of MTSEA, $k$ is the second order rate constant (in m$^{-1}$ s$^{-1}$), $C$ is the concentration of MTSEA (mM), and $t$ is the time (180 s). Fitting was achieved using the Enzfit software from BioSoft (Ferguson, MO).
mutant receptors exhibited high affinity binding for $^{125}$I-
[Sar$^1$,Ile$^8$]AngII except for D278C (7.29), D281C (7.32),
M284C(7.35), and P285C (7.36), which did not demonstrate any
detectable binding, and N295C(7.46), which showed a moderate
13-fold increase in $K_d$ (Table I). Those receptors where binding
was undetected were not used for SCAM analysis.

$B_{max}$ values for all detectable receptors ranged from 0.2- to 1.6-fold that of
the wild type receptor (Table I).

Effect of Extracellularly Added MTSEA on Binding Properties of Mutant Receptors
—To verify the location of reporter
cysteines introduced into TM7 of the receptor toward the bind-
ing pocket, all detectable receptor mutants were treated with
varying concentrations of MTSEA. MTSEA at 0.5 mM signifi-
cantly reduced ligand binding to 5 of the 20 cysteine substitu-
tion mutants tested (Fig. 3A). Increasing the MTSEA concen-
tration to 2 mM significantly reduced binding to seven mutant
receptors (Fig. 3B). Higher MTSEA concentrations (6 mM) did
not significantly modify the binding properties of the unaf-
fected cysteine mutant receptors (data not shown). Thus, Cys residues substituted for Ala 277(7.28), Val 280(7.31), Thr 282(7.33), Ala283(7.34), Ile 286(7.37), Ala 291(7.42), and Phe 301(7.52) conferred MTSEA sensitivity to the AT 1 receptor.

**Second Order Rate Constant of MTSEA Reactivity and Protection Assays**—To quantify the susceptibility of sensitive mutant receptors to MTSEA, we determined the second order rate constants (Table II). The most highly reactive cysteines were those substituted for Ala277(7.28) and Thr282(7.33) with constants of 2297 and 641 M⁻¹s⁻¹ respectively. The moderately reactive cysteines were those substituted for Val280(7.31), Ile286(7.37), Ala291(7.42), and Phe301(7.52) with values ranging from 3.4 to 14.2 M⁻¹s⁻¹. Cysteine substituted for Ala283(7.34) was the least reactive with a second order rate constant of 1.6 M⁻¹s⁻¹. To gain more insight about the location in the binding pocket of the reporter cysteines accessible to MTSEA, receptor mutants were preincubated with the ligand prior to MTSEA treatment. The cells were then submitted to an acid wash to dissociate the bound ligand, and receptors were then assayed for binding with the radiolabeled antagonist. Fig. 4 shows how preincubating with the antagonist [Sar¹,Ile⁸]AngII essentially protected the mutant receptors A277C(7.28), V280C(7.31), T282C(7.33), A283C(7.34), and I286C(7.37) from the effect of MTSEA with protection levels ranging from 65 to 90% (Fig. 4). More moderate protection from MTSEA was obtained for mutant receptors A291C(7.42) and F301C(7.52), which exhibited a 30% protection level.

**Altered Accessibility to TM7 Reporter Cysteines of a Constitutively Active AT₁ Receptor**—We made use of the constitutively active receptor, hAT₁-N111G (19), to assess and map the potentially altered accessibility of the engineered cysteines for MTSEA. As can be seen in Fig. 5, sensitivity of this receptor to 2 mM MTSEA is similar to that of the wild type receptor with a reduction of only 7% of total binding. Higher concentrations of

| Table II |
|-----------------|-----------------|
| MTSEA second order reaction rates to cysteine-substituted hAT₁ mutant receptors |
| The second order rate constant was determined as described under “Experimental Procedure.” The means and S.E. are shown for n independent experiments, each with triplicate determinations |
| k (M⁻¹s⁻¹) | n  |
| A277C | 2297 ± 558 | 3 |
| V280C | 14.2 ± 1.7 | 3 |
| T282C | 641 ± 94 | 3 |
| A283C | 1.6 ± 0.9 | 3 |
| I286C | 4.3 ± 0.5 | 3 |
| A291C | 6.2 ± 1.8 | 3 |
| F301C | 3.4 ± 0.7 | 3 |

Fig. 3. Effect of MTSEA on AT₁ mutant receptors bearing reporter cysteines in TM7. Intact COS-7 cells transiently expressing the wild type or mutants AT₁ receptors were incubated with freshly prepared 0.5 mM MTSEA (A) and 2 mM MTSEA (B) in a final volume of 0.2 ml at room temperature for 3 min. The reactions were stopped by dilution in ice-cold PBS. The intact cells were then incubated with 0.05 nM ¹²⁵I-[Sar¹,Ile⁸]AngII for 1.5 h at room temperature. The fractional inhibition was calculated as indicated under “Experimental Procedures.” The vertical line represents an arbitrary threshold that was set at 25% of binding inhibition to identify sensitive Cys mutant receptors. White bars indicate mutant receptors for which ligand binding was maintained up to 25% of the corresponding untreated receptor once reacted with MTSEA. Gray and black bars indicate mutants for which binding inhibition was higher than 25 and 50%, respectively, of the corresponding untreated receptor. The means and S.E. are shown. The results are representative of at least three separate experiments. WT, wild type.
PBS. The intact cells were then incubated with 0.05 nM 125I- AngII for 3 min. The reaction was stopped by dilution in ice-cold PBS. The intact COS-7 cells transiently expressing mutant receptors were incubated with freshly prepared MTSEA (0.5 mM) in a final volume of 0.2 ml at room temperature for 3 h. The cells were then washed with ice-cold PBS and incubated with 0.05 nM [Sar1,Ile8]AngII for 3 h at 16 °C. Protection was calculated as described under "Experimental Procedures." The means and S.E. are shown. The results are representative of at least three separate experiments.

**TABLE III**

| Receptor       | Kd (nM) | Bmax (pmol/mg) | n  |
|----------------|---------|----------------|----|
| N111G (Cys289, Cys290) | 0.7 ± 0.2 | 0.88 ± 0.25 | 5  |
| I276C          | no detectable binding | 3  |
| A277C          | 0.5 ± 0.2 | 0.42 ± 0.10 | 3  |
| D278C          | no detectable binding | 3  |
| I279C          | 0.4 ± 0.1 | 0.44 ± 0.10 | 3  |
| V290C          | 0.5 ± 0.1 | 0.56 ± 0.16 | 3  |
| D281C          | 0.6 ± 0.1 | 0.34 ± 0.10 | 3  |
| T282C          | 0.5 ± 0.1 | 0.32 ± 0.10 | 3  |
| A283C          | 0.5 ± 0.2 | 0.56 ± 0.12 | 3  |
| I286C          | no detectable binding | 3  |
| M284C          | no detectable binding | 3  |
| D281C (7.37)   | 0.7 ± 0.2 | 0.53 ± 0.12 | 3  |
| T287C          | 1.1 ± 0.3 | 0.63 ± 0.10 | 3  |
| I288C          | no detectable binding | 3  |
| I290C          | no detectable binding | 3  |
| A291C          | 1.3 ± 0.1 | 0.59 ± 0.10 | 3  |
| Y292C          | 0.8 ± 0.2 | 0.51 ± 0.17 | 3  |
| P293C          | 0.6 ± 0.1 | 0.55 ± 0.10 | 3  |
| N294C          | 1.5 ± 0.6 | 0.55 ± 0.15 | 3  |
| N295C          | 0.7 ± 0.2 | 0.94 ± 0.22 | 3  |
| L297C          | 0.5 ± 0.1 | 0.84 ± 0.31 | 3  |
| N298C          | 2.2 ± 0.8 | 1.25 ± 0.67 | 3  |
| P299C          | 2.2 ± 0.4 | 0.83 ± 0.26 | 3  |
| L300C          | 0.5 ± 0.1 | 0.65 ± 0.32 | 3  |
| F301C          | 0.4 ± 0.1 | 0.50 ± 0.10 | 3  |
| Y302C          | 0.8 ± 0.1 | 1.18 ± 0.10 | 3  |

**DISCUSSION**

The aim of this study, which relied on SCAM analysis, was to gain insight into the orientation of the residues of TM7 within the binding pocket of the AT1 receptor and to assess the struct-
Conformational Changes of Activated \( \text{AT}_1 \) Receptor

Tural mechanisms underlying receptor activation. Changes in ligand binding were monitored in a wild type receptor (ground state) background and compared with a constitutively active receptor. The SCAM method is based on the reactivity of engineered cysteines to MTSEA, a reagent that reacts a billion times faster with ionized cysteines than with the un-ionized thiol (13) and thus will covalently alkylate any cysteine located in a hydrophilic environment. Indeed lipid-exposed, buried, or disulfide-bonded cysteines are unlikely to ionize to a significant extent and hence are assumed to be unaffected by such modification induced by the MTS reagents. Two criteria were used to establish that an engineered cysteine was accessible in the binding site crevice: 1) MTSEA added extracellularly irreversibly inhibited ligand binding and 2) binding activity was protected from MTSEA by pretreatment with an AT1 receptor ligand.

On the basis of MTSEA insensitivity of the wild type receptor, it can be suggested that either endogenous cysteines are not alkylated by MTSEA or alklylation of reactive cysteines does not affect ligand binding. Four cysteine residues of the AT\(_1\) receptor are suspected to participate in disulfide bridges linking the N-terminal to ECL3 and ECL1 to ECL2 (20) and thus would not be alkylated. Other cysteines found within various TMs would either face the lipid environment of the cell membrane and be unavailable or would simply not be a determinant of the binding pocket conformation. Interestingly, a recent report has revealed that exposition of the AT\(_1\) receptor to MTSEA was able to inhibit ligand binding (10), whereas we did not observe significant binding loss. This discrepancy could be explained by the fact that prior to MTSEA treatment, Miura and Karnik (10) used a cell disruption method, which exposes both the intracellular and the extracellular domains of the receptor to the reagent. Using membrane preparations we also obtained binding inhibition after MTSEA treatment (data not shown). However, in the present study, our methodological approach of adding MTSEA on whole adherent cells expressing the AT\(_1\) receptor will expose only the extracellular ligand-accessible side of the receptor to MTSEA.

The 25 individual mutants incorporating reporter cysteines spanned residues 276–302. Molecular modeling of AT\(_1\) receptor TM7 by homology to the rhodopsin high resolution crystal structure, suggests that residues 276–281 are located in an extracellular domain as part of ECL3. MTSEA-sensitive Ala\(^{277}\) is positioned in a flexible domain rigidified by a disulfide bridge between the N-terminal tail and ECL3, whereas Val\(^{281}\) sits just at the border of TM7, which extends down to Leu\(^{305}\) (Fig. 7). Thr\(^{283}\) and Ile\(^{294}\) lie on the same helix face with appreciable exposure to a potential hydrophilic pocket, an exposition that seems to be facilitated by the presence of a helical deviation around Phe\(^{296}\).
Fig. 7. Molecular modeling of hAT1 receptor TM7 residues by homology to bovine rhodopsin. Schematic model of the AT1 receptor as generated by homology modeling showing the MTSEA-sensitive AT1 mutant receptors bearing reporter cysteines in TM7. Residues ranging from Ala277(7.28) to Tyr282(7.33) are shown. White residues represent Cys substitution that has yielded no inhibition following MTSEA treatment, whereas orange residues correspond to MTSEA sensitive substitutions identified by our SCAM analysis. Green residues represent a segment of the AT1 receptor encompassing helix 8.

and Asn294(7.45). Ala291(7.42) and Phe301(7.52) lie parallel to each other on the same helix face mostly facing the same area of the binding pocket. Ala291(7.42) is predicted to face the protein interior, a feature that seems to be highly conserved among GPCRs, because this position also points inside the binding pocket of the D2 dopamine receptor, M3 muscarinic receptor, A2 adenosine receptor, and rhodopsin (21–24). Phe301(7.52) is part of a classical α-helical segment underlying the conserved NPXY motif following the helical deviation.

The modeling also shows a regular accessibility pattern exposing one face of the helix to the binding pocket while leaving the other facing away (Fig. 7). Therefore, residues lying on the MTSEA-accessible face are more likely to be involved in ligand binding. Indeed, Phe293(7.44), a residue that constitutes a cross-linking site for a C-terminally modified analogue of AngII (25), points toward the same water-accessible crevice identified here (Fig. 7). Also, Phe301(7.52) has been suggested to form the binding pocket for nonpeptidic antagonists and peptide agonists (26). In contrast, residues that were not identified as being MTSEA-sensitive, such as Asn290(7.46) (27) and Tyr292(7.43) (28), have been shown to affect receptor activation rather than peptide binding and would therefore lie on the opposite face of the helix (Fig. 7). Furthermore, endogenous TM7 Cys residues (Cys289(7.40) and Cys286(7.47)) are located on the opposite, lipid-exposed face of the helix (Fig. 7), a feature that could be responsible for their poor reactivity with MTSEA as demonstrated in the present study.

Interestingly, most of the MTSEA-accessible residues that have been identified by our SCAM analysis (A277C(7.28), V280C(7.31), T282C(7.33), A283C(7.34), and I286C(7.37)) lie toward the top portion of the transmembrane domain, possibly close to the interface between lipid and the extracellular milieu (Fig. 7). This result can be partly explained by the fact that the residues necessary for an appropriate interaction between the ligand and the receptor are mainly located within this interface (29–31). Thus, these residues would form the top of the binding pocket of the receptor with the result that their alkylation with MTSEA may produce a steric hindrance, thereby impeding binding of the ligand. After identifying A291C(7.42) as a sensitive residue, we then obtained a stretch of nine unaffected residues before acquiring sensitivity with F301C(7.52). A possible explanation for the poor MTSEA-mediated effect on ligand binding between Ala291(7.42) and Phe301(7.52) comes from a possible modeling of the AT1 receptor (Fig. 7). When comparing TM7 to the accessibility pattern brought by the SCAM study, those nine unaffiliated residues perfectly superimpose with a conserved helical deviation at the center of the transmembrane domain. This helix deformation is consistent with observations made for rhodopsin (24) and has already been mapped by SCAM studies in TM7 of the D2 dopamine receptor (21). This particular structure probably lengthens the spatial proximity between residues Tyr292(7.43)_Leu300(7.51) and the ligand-containing binding crevice. It is therefore possible that 1) residues in the Tyr292(7.43)_Leu300(7.51) stretch do not react with MTSEA because of their relative inaccessibility from the binding pocket or 2) residues Tyr292(7.43)_Leu300(7.51) react with MTSEA but do not contribute in forming the binding pocket to disturb ligand binding. However, the first hypothesis seems more probable because we had previously shown that Phe8 of AngII interacts with Phe293(7.44) and Asn294(7.45) of the AT1 receptor (25), therefore supporting the contention that the C terminus of AngII dives deep inside the receptor-binding pocket to initiate agonist-mediated receptor activation.

Our data using protection assays and reaction rates of MTSEA also support the notion that specific residues within TM7 contribute to forming the binding pocket. Indeed, the most reactive and possibly very accessible Cys residues (A277C(7.28), V280C(7.31), and T282C(7.33)) were highly protected by the presence of the ligand, whereas the A291C(7.42) and F301C(7.52) mutants, although less reactive, remained nonetheless protected. However, protection does not mean that a particular residue makes contact with the ligand. We cannot rule out the possibility of indirect effects through propagated structural changes that would make a reporter Cys more or less accessible upon ligand binding.

To further probe into the mechanisms by which receptors undergo structural changes from the inactive to the active state, we took advantage of the constitutively active hAT1-N111G receptor. It is believed that the isomerization of conformers toward the active state, which involves transmembrane movement, is stabilized by agonist binding and would be mimicked in part by the constitutively active receptor (1, 33). Thus, we verified accessibility of TM7 residues within the structural background of the hAT1-N111G receptor to MTSEA and compared the pattern to the one obtained for the wild type receptor (Fig. 8). We found that those Cys residues that were unresponsive to MTSEA (in regard to binding inhibition) kept the same behavior in the N111G mutant receptor (Fig. 8).

Interestingly, those Cys residues (A277C(7.28), V280C(7.31), T282C(7.33), A283C(7.34), I286C(7.37), and F301C(7.52)) that have been shown to react with MTSEA in the wild type receptor and therefore affected ligand binding (Fig. 8A), were either much less or no longer reactive when engineered in the hAT1-N111G receptor background where constitutive activity was main-
Conformational Changes of Activated AT1 Receptor

Fig. 8. Helical wheel representation of TM7 reporter Cys residues and their pattern of reactivity to MTSEA. Positions in TM7 of MTSEA reacted Cys residues affecting [Sar1,Ile8]AngII binding are shown in a helical wheel representation viewed from the extracellular side for receptors with no additional mutation in TM3 (A) and for receptors in which Asn111(3.35) has been mutated for Gly in addition to reporter Cys in TM7 (B). Black closed circles correspond to Cys that have been shown in this study to inhibit 50% or more of 125I-[Sar1,Ile8]AngII binding when reacted with MTSEA, whereas gray closed circles indicate those that inhibited ligand binding at 25% or more. White circles indicate those mutant receptors that showed no effect on ligand binding when reacted with MTSEA or positions resulting in low or undetectable binding when substituted for Cys residues.

Fig. 9. Proposed structurally related activation mechanisms for the hAT1 receptor. Extracellular view of AT1 seven transmembrane domains based on the arrangement described for bovine rhodopsin (24). Residues that affect ligand binding once treated with MTSEA span a fixed area (~180°) of TM7 symbolized by a black-filled half-circle. The water-accessible crevice forming the binding pocket is suggested to be located between TMs 1, 2, 3, 5, 6, and 7. In the ground state, constraining intramolecular interactions help to keep the receptor in a basal state where functional coupling with G protein is kept at its minimum for the system to remain sensitive to ligand stimulation. Some of those constraining interactions in the case of the AT1 receptor are thought to be generated between TM2, TM3, and TM7 (32). Following agonist binding, intramolecular interactions are broken, and GPCRs go through a series of conformational rearrangements implicating TMs. In the present study we propose that TM7 could be excluded from the water-filled crevice forming the binding pocket following activation of the AT1 receptor.

The N111G-A291C(7.42) mutant exhibited a greatly induced reaction rate with MTSEA and an increased extent of binding inhibition after MTSEA reaction compared with the same mutant engineered in the wild type receptor background (Fig. 3 versus Fig. 6). A possible explanation might be that some helix rotation has occurred concomitantly with the proposed translation, therefore making the Cys39(7.42) side chain more exposed to the binding pocket in the N111G receptor. A similar translation/rotation movement has been documented for a constitutively active β2-adrenergic receptor and light-activated rhodopsin whereby a significant translation of TM3 occurs away from TM6 on the cytoplasmic receptor interface while both TMs rotate on the extracellular interface (4, 34–36). More recently, using the SCAM approach, a rigid body rotation of TM2 has been described for the constitutively activated AT1 receptor bearing mutations at residue Asn111(3.35) (10).

We further demonstrate that the MTSEA-mediated binding inhibition on a receptor mutant for which Phe301(7.52) has been substituted for Cys is greatly altered in a constitutively active mutant of the AT1 receptor when compared with the same mutation engineered in the wild type background (Figs. 6 and 3). The Phe301(7.52) is part of a highly conserved motif among the rhodopsin-like family of GPCRs, the 7.49NPXX7.53 motif, involved in activation of numerous GPCRs including the AT1 receptor (11, 37–39). However, its precise role in receptor activation is still obscure. It has been suggested that the NPXXY motif could be part of an intramolecular bonding network implicating interhelical interactions between TM2, TM3, and TM7. Indeed, the recently elucidated structure of bovine rhodopsin shows that the complex responsible for those interhelical interactions includes residues Gly120(3.35)–Asp83(2.50)–Asn302(7.49) (24, 40). Such a complex is well conserved in the AT1 receptor, implicating the corresponding residues Asn111(3.35)–Asp74(2.50)–Asn298(7.49). Studies have revealed that Asn111(3.35) could interact with Tyr292(4.43) in the basal state of the receptor and that AngII binding would favor new interactions between Asn111(3.35) and Tyr4 of AngII; Tyr292(4.43) would then be hydrogen-linked to Asp74(2.50) in the activated receptor state (41). Furthermore, mutations at Asn111(3.35) would impede the network between TM3 and TM7, resulting in a more relaxed receptor leading to constitutive activity (9). Our data on the altered behavior of the F301C(7.52) treated with MTSEA would support the idea that the N111G receptor achieves its constitutive activity partly by altering the NPXXY conformation.

Our data comparing the AT1 receptor ground state versus an activated state strongly point toward an altered spatial proximity of TM7 with regards to the binding pocket which exposes TM7 residues Ala277(7.28), Val280(7.31), Thr282(7.33), Ala283(7.34), Ile286(7.37), Ala290(7.42), and Phe301(7.52) to a water-accessible crevice. This movement of TM7 for an activated GPCR is reminiscent of recent observations on rhodopsin (5) and is thought to define an important structural mechanism supporting recep-
tor activation mediated by AngII. This could be a common feature found in numerous rhodopsin-like GPCRs.

REFERENCES

1. Gether, U., and Koblika, B. K. (1998) J. Biol. Chem. 273, 17979–17982
2. Dunham, T. D., and Farrens, D. L. (1999) J. Biol. Chem. 274, 1683–1690
3. Ghanouni, P., Steenhuis, J. J., Farrens, D. L., and Kobilka, B. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5997–6002
4. Rasmussen, S. G., Jensen, A. D., Liapakis, G., Ghanouni, P., Javitch, J. A., and Gether, U. (1999) Mol. Pharmacol. 56, 175–184
5. Altenbach, C., Cai, K., Klein-Seetharaman, J., Khorana, H. G., and Hubbell, W. L. (2001) Biochemistry 40, 15483–15492
6. Altenbach, C., Klein-Seetharaman, J., Cai, K., Khorana, H. G., and Hubbell, W. L. (2001) Biochemistry 40, 15493–15500
7. de Gasparo, M., Catt, K. J., Wright, J. W., and Unger, T. (2000) Pharmacol. Rev. 52, 415–472
8. Inoue, Y., Nakamura, N., and Inagami, T. (1997) J. Hypertens. 15, 703–714
9. Noda, K., Feng, Y. H., Liu, X. P., Saad, Y., Husain, A., and Karnik, S. S. (1996) Biochemistry 35, 16435–16442
10. Miura, S., and Karnik, S. S. (2002) J. Biol. Chem. 277, 24299–24305
11. Laporte, S. A., Servant, G., Richard, D. E., Escher, E., Guillemette, G., and Ledue, R. (1996) Mol. Pharmacol. 49, 89–95
12. Boucard, A. A., Wilkes, B. C., Laporte, S. A., Escher, E., Guillemette, G., and Ledue, R. (2000) Biochemistry 39, 9662–9670
13. Javitch, J. A., Shi, L., and Liapakis, G. (2002) Methods Enzymol. 343, 137–156
14. Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K. P., and Ernst, O. P. (1996) Biochem. J. 320, 829–838
15. Ballesteros, J. A., and Weinstein, H. (1995) Methods Neurosci. 25, 366–428
16. Boucard, A. A., Sauve, S. S., Guillemette, G., Escher, E., and Ledue, R. (2003) Biochem. J. 370, 829–838
17. Javitch, J. A., Li, X., Kaback, J., and Karlin, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10355–10359
18. Dauber-Osguthorpe, P., Roberts, V. A., Osguthorpe, D., Wolff, J., Genest, M., and Hagler, A. T. (1988) Proteins 4, 31–47
19. Feng, Y. H., and Karnik, S. S. (1999) J. Biol. Chem. 274, 35546–35552
20. Ohyanagi, K., Yamato, Y., Sano, T., Nakagomi, Y., Hamakubo, T., Morishima, I., and Inagami, T. (1995) Regul. Pept. 57, 141–147
21. Fu, D., Ballesteros, J. A., Weinstein, H., Chen, J., and Javitch, J. A. (1996) Biochemistry 35, 11278–11285
22. Hamdan, F. F., Ward, S. D., Siddiqui, N. A., Bloodworth, L. M., and Wess, J. (2002) Biochemistry 41, 7647–7658
23. Dawson, E. S., and Wells, J. N. (2001) Mol. Pharmacol. 59, 1187–1195
24. Palczewski, K., Kumasaka, T., Horie, T., Behlke, C. A., Motoshiba, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
25. Perordin, J., Deraet, M., Auger-Messier, M., Boucard, A. A., Rihakova, L., Beaulieu, M. E., Lavigne, P., Parent, J. L., Guillemette, G., Ledue, R., and Escher, E. (2002) Biochemistry 41, 14348–14356
26. Hunyady, L., Bor, M., Bauckl, A. J., Balla, T., and Catt, K. J. (1995) J. Biol. Chem. 270, 16602–16609
27. Hunyady, L., Ji, H., Jagadeesh, G., Zhang, M., Gaborik, Z., Mihalik, B., and Catt, K. J. (1998) Mol. Pharmacol. 54, 427–434
28. Marie, J., Maigret, B., Joseph, M. P., Largueru, R., Notu, S., Lombard, C., and Bonnafous, J. C. (1994) J. Biol. Chem. 269, 20815–20818
29. Hunyady, L., Balla, T., and Catt, K. J. (1996) Trends Pharmacol. Sci. 17, 135–140
30. Hjorth, S. A., Schambży, H. T., Greenlee, W. J., and Schwartz, T. W. (1994) J. Biol. Chem. 269, 30953–30959
31. Le, M. T., Vanderheyden, P. M., Strazak, M., Huyndaey, L., and Vasquelin, G. (2002) J. Biol. Chem. 277, 23107–23110
32. Miura, S., Zhang, J., Baros, J., and Karnik, S. S. (2003) J. Biol. Chem. 278, 3720–3725
33. Seifert, R., and Wenzel-Seifert, K. (2002) Naunyn-Schmiedeberg's Arch. Pharmacol. 366, 381–416
34. Javitch, J. A., Fu, D., Liapakis, G., and Chen, J. (1997) J. Biol. Chem. 272, 18546–18549
35. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770
36. Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Nature 383, 347–350
37. Fritz, O., Filip, S., Kuksa, V., Palczewski, K., Hofmann, K. P., and Ernst, O. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2290–2295
38. Gales, C., Kowalski-Chauvel, A., Dufour, M. N., Seva, C., Moroder, L., Pradayrol, L., Vuyyse, N., Fourmy, D., and Silvente-Poiret, S. (2000) J. Biol. Chem. 275, 17321–17327
39. Priebeau, C., Visier, I., Ebersole, B. J., Weinstein, H., and Sealfon, S. C. (2002) J. Biol. Chem. 277, 36577–36584
40. Menon, S. T., Han, M., and Sakmar, T. P. (2001) Physiol. Rev. 81, 1659–1688
41. Joseph, M. P., Maigret, B., Bonnafous, J. C., Marie, J., and Scheraga, H. A. (1995) J. Protein Chem. 14, 381–398
Constitutive Activation of the Angiotensin II Type 1 Receptor Alters the Spatial Proximity of Transmembrane 7 to the Ligand-binding Pocket
Antony A. Boucard, Marise Roy, Marie-Eve Beaulieu, Pierre Lavigne, Emanuel Escher, Gaétan Guillemette and Richard Leduc

J. Biol. Chem. 2003, 278:36628-36636.
doi: 10.1074/jbc.M305952200 originally published online July 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305952200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 22 of which can be accessed free at http://www.jbc.org/content/278/38/36628.full.html#ref-list-1