The Second Transmembrane Domain of the Human Type 1 Angiotensin II Receptor Participates in the Formation of the Ligand Binding Pocket and Undergoes Integral Pivoting Movement during the Process of Receptor Activation*

Received for publication, October 22, 2008, and in revised form, February 26, 2009. Published, JBC Papers in Press, March 9, 2009, DOI 10.1074/jbc.M808113200

Ivana Domazet, Brian J. Holleran, Stéphane S. Martin, Pierre Lavigne1, Richard Leduc2, Emanuel Escher3, and Gaétan Guillemette4

From the Department of Pharmacology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada

The octapeptide hormone angiotensin II (AngII) exerts a wide variety of cardiovascular effects through the activation of the angiotensin II type-1 (AT1) receptor, which belongs to the G protein-coupled receptor superfamily. Like other G protein-coupled receptors, the AT1 receptor possesses seven transmembrane domains that provide structural support for the formation of the ligand-binding pocket. In order to identify those residues in the second transmembrane domain (TMD2) that contribute to the formation of the binding pocket of the AT1 receptor, we used the substituted cysteine accessibility method. All of the residues within the Leu-70 to Trp-94 region were mutated one at a time to a cysteine, and, after expression in COS-7 cells, the mutant receptors were treated with the sulphydryl-specific alkylating agent methanethiosulfonate-ethylammonium (MTSEA). MTSEA reacts selectively with water-accessible, free sulfhydryl groups of endogenous or introduced point mutation cysteines. If a cysteine is found in the binding pocket, the covalent modification will affect the binding kinetics of the ligand. MTSEA substantially decreased the binding affinity of D74C-AT1, L81C-AT1, A85C-AT1, T88C-AT1, and A89C-AT1 mutant receptors, which suggests that these residues orient themselves within the water-accessible binding pocket of the AT1 receptor. Interestingly, this pattern of acquired MTSEA sensitivity was altered for TMD2 reporter cysteines engineered in a constitutively active N111G-AT1 receptor background. Indeed, mutant D74C-N111G-AT1 became insensitive to MTSEA, whereas mutant L81C-N111G-AT1 lost some sensitivity and mutant V86C-N111G-AT1 became sensitive to MTSEA. Our results suggest that constitutive activation of the AT1 receptor causes TMD2 to pivot, bringing the top of TMD2 closer to the binding pocket and pushing the bottom of TMD2 away from the binding pocket.

*This work was supported by the Canadian Institutes of Health Research. This article was submitted to fulfill the requirements of a Ph.D. thesis for I. D. at the Université de Sherbrooke.

1 A Chercheur Senior of the Fonds de la recherche en Santé du Québec.
2 A Chercheur National of the Fonds de la recherche en Santé du Québec.
3 Recipient of the J.C. Edwards Chair in Cardiovascular Research.
4 To whom correspondence should be addressed: Dept. of Pharmacology, Faculty of Medicine, Université de Sherbrooke, 3001 12th Ave. N., Sherbrooke, Quebec J1H 5N4, Canada. Tel.: 819-564-5347; Fax: 819-564-5400; E-mail: Gaetan.Guillemette@USherbrooke.ca.

The octapeptide hormone angiotensin II (AngII) is the active component of the renin-angiotensin system. It exerts a wide variety of physiological effects, including vascular contraction, aldosterone secretion, neuronal activation, and cardiovascular cell growth and proliferation (1). Virtually all of the known physiological effects of AngII are produced through the activation of the AT1 receptor, which belongs to the G protein-coupled receptor (GPCR) superfamily (2, 3). GPCRs possess seven transmembrane domains (TMD), which provide structural support for signal transduction. The AT1 receptor interacts with the G protein Gq/11, which activates a phospholipase C, which in turn generates inositol 1,4,5-trisphosphate and diacylglycerol from the cleavage of phosphatidylinositol 4,5-bisphosphate (4, 5). Inositol 1,4,5-trisphosphate causes the release of Ca2+ from an intracellular store, whereas diacylglycerol activates protein kinase C.

Like other GPCRs, the AT1 receptor undergoes spontaneous isomerization between its inactive state (favored in the absence of agonist) and its active state (induced or stabilized by the agonist) (6). Movement of TMD helices through translational or rotational displacement is believed to be essential to achieve the active state (7, 8). It has been proposed that TMD3, TMD5, TMD6, and TMD7 may participate in the activation process of the AT1 receptor by providing a network of interactions through the AngII-binding pocket (9). The dynamics of this network are thought to be modified following agonist binding, thereby forcing the receptor to form new interactions between the TMDs.

Based on homology with the high resolution structure of rhodopsin, the archetypal GPCR (10), it was expected that the binding site of the AT1 receptor would involve the seven mostly hydrophobic TMDs and would be accessible to charged water-soluble ligands, like AngII. For this receptor, the binding site would thus be contained within a water-accessible crevice, the binding pocket, extending from the extracellular surface of the receptor to the transmembrane portion. Using a photoaffinity labeling approach, we directly

5 The abbreviations used are: AngII, angiotensin II; AT1, angiotensin II type-1; GPCR, G protein-coupled receptor; TMD, transmembrane domain; SCAM, substituted cysteine accessibility method; MTSEA, methanethiosulfonate-ethylammonium; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline.
identified ligand-contact points within the second extracellular loop and the seventh TMD of the AT1 receptor (11–13). Interestingly, numerous mutagenesis studies have provided the basis for a model in which an interaction between Asn-111 in TMD3 and Tyr-292 in TMD7 maintains the AT1 receptor in the inactive conformation. The agonist AngII would disrupt this interaction and promote the active conformational state (14). In support of this model, it was further shown that substitution of Asn-111 for a residue of smaller size (Ala or Gly) confers constitutive activity on the AT1 receptor (15–17).

The substituted cysteine accessibility method (SCAM) (18–20) is an ingenious approach for systematically identifying the residues in a TMD that contribute to the binding site pocket of a GPCR. Consecutive residues within TMDs are mutated to cysteine, one at a time, and the mutant receptors are expressed in heterologous cells. If ligand binding to a cysteine-substituted mutant is unchanged compared with wild-type receptor, it is assumed that the structure of the mutant receptor, especially around the binding site, is similar to that of wild type and therefore that the substituted cysteine lies in an orientation similar to that of the wild-type residue. In TMDs, the sulfhydryl of a cysteine oriented toward the binding site pocket should react faster with a positively charged sulfhydryl reagent like methanethiosulfonate-ethylammonium (MTSEA) than sulfhydryls facing the interior of the protein or the lipid bilayer. Two criteria are used for identifying engineered cysteines on the surface of the binding site pocket: (i) the reaction with MTSEA alters binding irreversibly, and (ii) the reaction is retarded by the presence of ligand. We previously used this approach to identify the residues in TMD3, TMD6, and TMD7 that form the surface of the binding site pocket in the wild-type AT1 receptor and in the constitutively active N111G-AT1 receptor (21–23). Here we report the application of SCAM to probe TMD2 in the wild-type and constitutively active receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, bacitracin, and soybean trypsin inhibitor were from Sigma. The sulfhydryl-specific alkylating reagent MTSEA (CH3SO2-SCH2.CH2.NH3+) was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). The cDNA clone for the human AT1 receptor subcloned in the mammalian expression vector pcDNA3 was kindly provided by Dr. Sylvain Meloche (Université de Montréal). LipofectAmine2000® and culture media were obtained from Invitrogen. 125I-[Sar1,Ile8]AngII (specific radioactivity ~1500 Ci/mmole) was prepared with IODO-GEN® (Perbio Science, Erembodegem, Belgium) according to the method of Fraker and Speck (24) and as previously reported (25).

**Numbering of Residues in TMD2**—Residues in TMD2 of the human AT1 receptor were given two numbering schemes. First, residues were numbered according to their positions in the human AT1 receptor sequence. Second, residues were also indexed according to their position relative to the most conserved residue in the TMD in which it is located (26). By definition, the most conserved residue was assigned the position index “50” (e.g. in TMD2, Asp-74 is the most conserved residue and was designated Asp-74(2.50)), whereas the upstream residue was designated Leu-75(2.51) and the downstream residue was designated Ala-73(2.49). This indexing simplified the identification of aligned residues in different GPCRs.

**Oligodeoxynucleotide Site-directed Mutagenesis**—Site-directed mutagenesis was performed on the wild-type AT1 receptor with the overlap PCR method (Expand High Fidelity PCR System; Roche Applied Science). Briefly, forward and reverse oligonucleotides were constructed to introduce cysteine mutations between Leu-70(2.40) and Trp-94(2.70). PCR products were subcloned into the HindIII-XbaI sites of the mammalian expression vector pcDNA3.1. Site-directed mutations were then confirmed by automated DNA sequencing by aligning the AT1 sequence with multiAlin (26).

**Cell Culture and Transfection**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM l-glutamine and 10% (v/v) fetal bovine serum. The cells were seeded into 100-mm culture dishes at a density of 2 × 10⁶ cells/dish. When the cells reached ~90% confluence, they were transfected with 4 μg of plasmid DNA and 15 μl of lipofectAmine2000®. After 24 h, transfected cells were trypsinized, distributed into 12-well plates, and grown for an additional 24 h in complete DMEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin before the MTSEA treatment and binding assay.

**Binding Experiments**—COS-7 cells were grown for 36 h post-transfection in 100-mm culture dishes, washed once with phosphate-buffered saline (PBS), and subjected to one freeze-thaw cycle. Broken cells were then gently scraped into washing buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl2), 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 MgCl2, 0.1% bovine serum albumin, 0.01% bacitracin, 0.01% soybean trypsin inhibitor). Saturation binding experiments were done by incubating broken cells (20–40 μg of protein) for 1 h at room temperature with increasing concentrations of 125I-[Sar1,Ile8]AngII in a final volume of 500 μl. Nonspecific binding was determined in the presence of 1 μM unlabeled [Sar1,Ile8]AngII. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked for at least 3 h in binding buffer. Receptor-bound radioactivity was evaluated by γ-counting.

**Treatment with MTSEA**—MTSEA treatment was performed according to the procedure of Javitch et al. (18), with minor modifications. Two days after transfection, the cells, which were grown in 12-well plates, were washed with PBS and incubated for 3 min at room temperature with freshly prepared MTSEA at the desired concentrations (typically from 0.5 to 6 mM) in a final volume of 0.2 ml. The reaction was stopped by washing the cells with ice-cold PBS. Intact cells were then incubated in binding medium (DMEM, 25 mM HEPES, 0.1% bovine serum albumin, pH 7.4) containing 0.05 mM 125I-[Sar1,Ile8]AngII for 90 min at room temperature. 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 fractional binding inhibition was calculated as \((1 - (\text{specific binding after the MTSEA treatment}/\text{specific binding without the treatment})) \times 100\).

**Protection against MTSEA Reaction by \([\text{Sar}^{1},\text{Ile}^{8}]\text{AngII}\)—**

Transfected cells grown in 12-well plates were washed once with PBS and incubated in the presence or absence of 100 nM \([\text{Sar}^{1},\text{Ile}^{8}]\text{AngII}\) for 1 h at 16°C (to avoid internalization of receptors). The cells were washed to remove excess ligand and then treated with MTSEA. The cells were washed three times with ice-cold PBS and once with an acidic buffer (150 mM NaCl, 50 mM acetic acid, pH 3.0) to dissociate bound ligand. They were then incubated for 3 h at 16°C in binding medium (DMEM, 25 mM HEPES, 0.1% bovine serum albumin, pH 7.4) containing 0.05 nM \(^{125}\text{I}-[\text{Sar}^{1},\text{Ile}^{8}]\text{AngII}\). The percentage of protection was calculated as \(((\text{inhibition in the absence of } [\text{Sar}^{1},\text{Ile}^{8}]\text{AngII})/(\text{inhibition in the presence of } [\text{Sar}^{1},\text{Ile}^{8}]\text{AngII}))\) × 100.

**Phospholipase C Assay—**

Inositol phosphate accumulation was determined as described previously (27). In brief, COS-7 cells were seeded in 6-well plates, transfected, and labeled for 16 h in serum-free M199 containing 10 μCi/ml \(^{3}H\)-inositol (MP Biomedicals, Solon, OH). Cells were washed twice with PBS plus 0.1% (w/v) dextrose and then incubated in stimulation buffer (DMEM containing 25 mM HEPES, 10 mM LiCl, and 0.1% bovine serum albumin, pH 7.4) for 30 min at 37°C. Incubations were terminated by the addition of ice-cold perchloric acid (final concentration, 5% (v/v)). Water-soluble inositol phosphates were then extracted with an equal volume of a 1:1 (v/v) mixture of 1,1,2-trichlorotrifluoroethane and tri-N-octylamine. The samples were mixed vigorously and centrifuged at 2500 \(\times\) g for 30 min. The upper phase

---

**TABLE 1**

**Binding Properties of \([\text{Sar}^{1},\text{Ile}^{8}]\text{AngII}\) to cysteine-substituted hAT\(_{1}\) mutant receptors**

Cells transfected with the appropriate receptor were assayed as described under “Experimental Procedures.” Binding affinities (\(K_d\)) and maximal binding capacities (\(B_{\text{max}}\)) are expressed as the means ± S.D. of values obtained in \(n\) independent experiments performed in duplicate. Mutants P82C, W84C, Y87C, Y92C, and W94C did not demonstrate any detectable binding.

|          | \(K_d\) (nM) | \(B_{\text{max}}\) (fmol/mg) | n     |
|----------|--------------|-------------------------------|-------|
| Wild type (Cys-76) | 0.6 ± 0.3    | 4282 ± 903   | 11    |
| L70C    | 0.7 ± 0.2    | 1428 ± 796   | 4     |
| A71C    | 0.9 ± 0.3    | 2729 ± 523   | 3     |
| L72C    | 0.4 ± 0.2    | 1923 ± 549   | 4     |
| A73C    | 0.4 ± 0.1    | 1814 ± 309   | 3     |
| D74C    | 1.2 ± 0.7    | 1141 ± 320   | 3     |
| L75C    | 0.9 ± 0.4    | 1405 ± 232   | 3     |
| F77C    | 3.1 ± 0.6    | 2256 ± 478   | 3     |
| L78C    | 0.4 ± 0.1    | 1412 ± 300   | 3     |
| L79C    | 0.5 ± 0.2    | 1833 ± 1020  | 3     |
| T80C    | 0.3 ± 0.0    | 1918 ± 711   | 3     |
| L81C    | 4.0 ± 1.3    | 2415 ± 872   | 3     |
| L83C    | 1.8 ± 0.2    | 1288 ± 691   | 3     |
| A85C    | 0.9 ± 0.3    | 1074 ± 405   | 3     |
| V86C    | 0.6 ± 0.3    | 1709 ± 140   | 3     |
| T88C    | 3.2 ± 1.9    | 5633 ± 72    | 3     |
| A89C    | 0.5 ± 0.1    | 1061 ± 451   | 3     |
| M90C    | 1.6 ± 1.0    | 490 ± 20     | 3     |
| E91C    | 2.1 ± 0.6    | 1622 ± 950   | 3     |
| R93C    | 0.8 ± 0.3    | 1152 ± 67    | 3     |

---

**FIGURE 1.** Schematic representation of the human AT\(_{1}\) receptor. The numbers indicate the positions of cysteines and other residues in the receptor. The gray closed circles represent cysteine residues that are thought to be linked via disulfide bridges, and the black closed circles represent cysteine residues whose side chains do not form a disulfide bridge. Mutated TMD2 residues are located between Leu\(^{70}\) and Trp\(^{94}\) inclusively. Potential N-glycosylation sites (Asn\(^{4}\), Asn\(^{176}\), and Asn\(^{18}\)) are indicated. Asn\(^{111}\) in TMD3 is also shown in gray.
containing inositol phosphates was applied to an AG1-X8 resin column (Bio-Rad). Inositol phosphates were eluted sequentially by the addition of an ammonium formate/formic acid solution of increasing ionic strength. Fractions containing inositol phosphates were collected and measured in a liquid scintillation counter.

Data Analysis—Results are presented as means ± S.D. Specific binding data ($B_{max}$ and $K_d$) were analyzed with Prism version 4.0 for Windows (GraphPad Software, San Diego CA), using a one-site binding hyperbola nonlinear regression analysis.

RESULTS

Binding Properties of Mutant Receptors Bearing Cysteines in TMD2—To identify the residues in TMD2 that face the binding site pocket of the AT$_1$ receptor, we mutated 24 consecutive residues between Leu-70(2.47) and Trp-94(2.70) to cysteine, one at a time. Each mutant receptor was transiently expressed in COS-7 cells. To assess the conservation of the global conformation of these receptors after the substitution, pharmacological parameters describing the equilibrium binding of the radiolabeled competitive ligand $^{125}$I-[Sar$^1$,Ile$^8$]AngII, such as $K_d$ and $B_{max}$ were determined (Table 1). All mutant AT$_1$ receptors exhibited high binding affinity for $^{125}$I-[Sar$^1$,Ile$^8$]AngII (similar to that of the wild-type AT$_1$ receptor) except for mutants P82C(2.58), W84C(2.60), Y87C(2.63), Y92C(2.68), and W94C(2.70), which did not demonstrate any detectable binding activity and were not used for the SCAM analysis. $B_{max}$ values for all detectable receptors ranged from 490 to 4282 fmol/mg of protein.

FIGURE 2. MTSEA treatment of the wild-type AT$_1$ receptor and sensitive reporter cysteine-bearing mutant receptors. Intact COS-7 cells transiently expressing wild-type, D74C, L81C, A85C, T88C, or A89C AT$_1$ receptors were incubated for 3 min at room temperature with increasing concentrations of freshly prepared MTSEA (0.5–6 mM). The intact cells were then incubated for 90 min at room temperature with 0.05 nM $^{125}$I-[Sar$^1$,Ile$^8$]AngII, and their binding properties were evaluated as indicated under “Experimental Procedures.” Each curve represents the means ± S.D. of data obtained from at least three independent experiments.

Effect of Extracellularly Added MTSEA on the Binding Properties of Mutant Receptors—To verify whether the reporter cysteines introduced into the TMD2 of the AT$_1$ receptor were oriented toward the binding pocket, mutant receptors were treated with concentrations of MTSEA varying between 0.5 and 6 mM. We also verified whether the wild-type AT$_1$ receptor, which contains 10 endogenous cysteines (Fig. 1), was sensitive to MTSEA treatment. Fig. 2 shows that various concentrations of MTSEA had very little effect (no more than a 25% reduction) on the binding properties of the wild-type AT$_1$ receptor, indicating that the endogenous cysteines made a relatively small contribution to the binding site pocket. A 3-min treatment with 0.5 mM MTSEA (Fig. 3A) strongly inhibited the binding properties of mutants L81C(2.57)-AT$_1$ (binding inhibition of 65%), A85C(2.61)-AT$_1$ (binding inhibition of 60%), and T88C(2.64)-AT$_1$ (binding inhibition of 75%), whereas it had only a minor effect on the binding properties of the other mutant receptors. At higher MTSEA concentrations (2 mM and above), the binding properties of mutant receptors D74C(2.50)-AT$_1$ and A89C(2.65)-AT$_1$ were also slightly affected (Fig. 3B). Overall, the most reactive cysteines were those substituted for L81C(2.57), A85C(2.61), and T88C(2.64) whereas the cysteine substituted for D74C(2.50) and A89C(2.65) were less reactive.

Altered Accessibility to TMD2 Reporter Cysteines in the Constitutively Active N111G-AT$_1$ Receptor

FIGURE 3. Effects of MTSEA on different mutant AT$_1$ receptors bearing a reporter cysteine in TMD2. Intact COS-7 cells transiently expressing wild-type or mutant AT$_1$ receptors were incubated for 3 min at room temperature with freshly prepared 0.5 mM MTSEA (A) or 2 mM MTSEA (B). The intact cells were then incubated for 90 min at room temperature with 0.05 nM $^{125}$I-[Sar$^1$,Ile$^8$]AngII. The percentage of binding inhibition was calculated using an arbitrary threshold used to identify cysteine-sensitive mutants and was set at a value corresponding to binding inhibition 20% greater than the value for the wild-type AT$_1$ receptor. The white bars indicate mutant receptors for which binding activities were not appreciably reduced compared with the wild-type receptor after treatment with MTSEA. The gray and black bars indicate mutant receptors for which binding activities were slightly reduced (gray) or strongly reduced (black) after treatment with MTSEA. Each bar represents the means ± S.D. of data from at least three independent experiments.
SCAM Method to Study the Ligand-binding Pocket of the hAT1 Receptor

TABLE 2

| Binding properties of [Sar1, Ile8]AngII to cysteine-substituted hAT1 mutant receptors bearing the N111G mutation |
|------------------------------------------------------|
| Cells transiently expressing the N111G-AT1, L81C-N111G-AT1, A85C-N111G-AT1, Y92C-N111G-AT1, and W94C-N111G did not demonstrate any detectable binding. |

Cells transfected with the appropriate receptor were assayed as described under "Experimental Procedures." Binding affinities (Kd) and maximal binding capacities (Bmax) are expressed as the means ± S.D. of values obtained in n independent experiments performed in duplicate. Mutants P82C-N111G, L83C-N111G, W84C-N111G, Y87C-N111G, Y92C-N111G, and W94C-N111G did not demonstrate any detectable binding.

| Kd (mM) | Bmax (fmol/mg) | n |
|--------|---------------|---|
| 0.8 ± 0.2 | 3003 ± 885 | 10 |
| 3.6 ± 1.6 | 3087 ± 235 | 3 |
| 1.3 ± 0.2 | 2787 ± 777 | 3 |
| 0.9 ± 0.6 | 2688 ± 877 | 7 |
| 0.6 ± 0.1 | 3967 ± 1516 | 4 |
| 1.8 ± 0.1 | 1781 ± 775 | 3 |
| 0.6 ± 0.0 | 2336 ± 689 | 3 |
| 0.7 ± 0.0 | 3004 ± 213 | 3 |
| 0.6 ± 0.1 | 1645 ± 704 | 3 |
| 0.5 ± 0.0 | 1868 ± 280 | 3 |
| 0.4 ± 0.0 | 3395 ± 456 | 3 |
| 1.7 ± 0.2 | 1780 ± 252 | 2 |
| 0.9 ± 0.1 | 622 ± 429 | 3 |
| 2.6 ± 0.6 | 570 ± 202 | 3 |
| 0.7 ± 0.1 | 819 ± 608 | 3 |
| 1.0 ± 0.4 | 1019 ± 854 | 3 |
| 2.9 ± 1.0 | 980 ± 492 | 3 |
| 2.1 ± 0.6 | 1622 ± 950 | 3 |
| 1.2 ± 0.5 | 1175 ± 75 | 3 |

FIGURE 4. MTSEA treatment of the N111G-AT1 receptor and sensitive reporter cysteine-bearing mutant N111G-AT1 receptors. Intact COS-7 cells transiently expressing the N111G-AT1, L81C-N111G-AT1, A85C-N111G-AT1, V86C-N111G-AT1, T88C-N111G-AT1, or W94C-N111G-AT1 receptors were incubated for 3 min at room temperature with increasing concentrations of MTSEA (0.5–6 mM). The intact cells were then incubated for 90 min at room temperature with 0.05 nM [Sar1, Ile8]AngII, and their binding properties were evaluated as indicated under "Experimental Procedures." Each curve represents the means ± S.D. of data from at least three independent experiments.

DISCUSSION

The rationale of this study, which relied on SCAM analysis, was to gain an insight into the orientation of TMD2 of the AT1 receptor by identifying the residues accessible to MTSEA within the binding site pocket. Mapping these residues in the ground state receptor and the constitutively active N111G
SCAM Method to Study the Ligand-binding Pocket of the hAT1 Receptor

FIGURE 5. Effect of MTSEA on different mutant N111G-AT1 receptors bearing a reporter cysteine in TMD2. Intact COS-7 cells transiently expressing the mutant N111G-AT1 receptors were incubated for 3 min at room temperature with freshly prepared 0.5 mM MTSEA (A) or 2 mM MTSEA (B). The intact cells were then incubated for 90 min at room temperature with 0.05 nM 125I-[Sar1,Ile8]AngII. Protection was calculated as described under “Experimental Procedures.” The vertical line represents an arbitrary threshold used to identify cysteine-sensitive mutants. It was set at a value corresponding to binding inhibition 20% greater than the value for the N111G-AT1 receptor.

FIGURE 6. [Sar1,Ile8]AngII protection of MTSEA-sensitive mutant receptors. Intact COS-7 cells transiently expressing MTSEA-sensitive mutant AT1 receptors were preincubated for 1 h at 16 °C in the absence or presence of 100 nM [Sar1,Ile8]AngII with optimal MTSEA concentrations to achieve maximal binding inhibition of each receptor. The MTSEA concentrations were as follows: 0.5 mM for L83C-AT1, A85C-AT1, T88C-AT1, A85C-N111G-AT1, V86C-N111G-AT1, and T88C-N111G-AT1; 2 mM for D74C-AT1, A89C-AT1, L70C-AT1, A73C-TMD2, D74C-TMD2, L75C-AT1, F77C-AT1, L76C-AT1, L79C-AT1, T80C-AT1, A85C-AT1, A89C-AT1, V86C-AT1, T88C-AT1, A85C-N111G-AT1, A89C-N111G-AT1, V86C-N111G-AT1, T88C-N111G-AT1, L81C-N111G-AT1, A85C-N111G-AT1, A89C-N111G-AT1, V86C-N111G-AT1, T88C-N111G-AT1, M90C-AT1, E91C-AT1, and R93C-AT1. Protection was calculated as described under “Experimental Procedures.” Each bar represents the means ± S.D. of data from at least three independent experiments.

SCAM Method to Study the Ligand-binding Pocket of the hAT1 Receptor

state AT1 receptor or the constitutively active N111G-AT1 receptor (Fig. 7).

As previously reported, the insensitivity of the wild-type receptor to MTSEA suggests either that endogenous cysteines are not alkylated by MTSEA or that their alkylation does not affect the binding of the ligand (23). Our methodological approach of adding the MTSEA reagent to whole adherent cells expressing the AT1 receptor essentially exposed only the extracellular ligand-accessible side of the receptor to MTSEA. Interestingly, most of the MTSEA-accessible residues that we identified with the SCAM approach lie in the middle (D74C(2.50), L81C(2.57)) to the top portion of TMD2 (A85C(2.61), T88C(2.64), A89C(2.65)) (Fig. 8). These results imply that the residues involved in the interaction with the ligand are mostly located within this interface. Thus, residue Ala-89(2.65) would delineate the top of the binding pocket, whereas residue Asp-74(2.50) would delineate the bottom of the water-accessible binding pocket of the receptor. Along with these residues, three other residues, Leu-81(2.57), Ala-85(2.61), and Thr-88(2.64), would lie on the same α-helix face in the ground state of the receptor, with great exposure to a potential hydrophilic pocket (see Fig. 8). By a mechanism that could be steric, electrostatic, or indirect, the alkylation of these residues with MTSEA would hamper the binding of the ligand. It is very likely that these two subsets of residues do not interact with the same portion of the ligand. Furthermore, it is assumed that water-accessible residues are in the binding site pocket if a competitive ligand protects them from the effect of MTSEA. The competitive ligand [Sar1,Ile8]AngII protected all of the residues tested, thus supporting the notion that these specific residues within TMD2 are located in the binding pocket. Although the mutant L83C-AT1 did show sensitivity (Fig. 3B), we did not consider residue Leu-83(2.59) to be in the binding pocket, because 1) it is not on the same helical face as the other MTSEA-sensitive residues; 2) in the constitutively active background, the mutant L83C(2.59)-N111G-AT1 did not show any binding activity. These results suggest that residue Leu-83(2.59) is important to maintain the global conformation of the receptor.

Our finding that residues Asp-74(2.50), Leu-81(2.57), Ala-85(2.61), Val-86(2.62), Thr-88(2.64), and Ala-89(2.65) are in the locating the binding pocket of the AT1 receptor is in accordance with the current models proposed for bovine rhodopsin (10), squid rhodopsin (28), the opsin receptor (29), and the dopamine D2 receptor (30). Indeed, residue Thr-94(2.61) is thought to be one of the contact points with retinal in the crystal struc-
The SCAM method was used to study the ligand-binding pocket of the hAT1 receptor. Transfected COS-7 cells were preloaded for 16–24 h with 10 μCi/ml [myo-3H]inositol. Inositol phosphates (sum of inositol bisphosphate, inositol trisphosphate, and inositol tetrakisphosphate) accumulated within a period of 30 min were determined as described under "Experimental Procedures." These results represent the mean ± S.D. values from three independent experiments (done in triplicate), where inositol phosphate production was normalized for incorporation of [myo-3H]inositol into phospholipids and for receptor expression level (determined by saturation binding assays).

FIGURE 7. Basal levels of inositol phosphates in cells expressing the wild type and mutant AT1 receptors. Transfected COS-7 cells were preloaded for 16–24 h with 10 μCi/ml [myo-3H]inositol. Inositol phosphates (sum of inositol bisphosphate, inositol trisphosphate, and inositol tetrakisphosphate) accumulated within a period of 30 min were determined as described under “Experimental Procedures.” These results represent the mean ± S.D. values from three independent experiments (done in triplicate), where inositol phosphate production was normalized for incorporation of [myo-3H]inositol into phospholipids and for receptor expression level (determined by saturation binding assays).

FIGURE 8. Helical wheel representation of TMD2 reporter cysteines and their pattern of reactivity to MTSEA. A and B, positions in TMD2 of MTSEA-alkylated cysteines affecting ligand binding are shown in a helical wheel representation viewed from the extracellular side for receptors with no additional mutation in TMD2 (A) and for receptors in which Asn-111 has been mutated to Gly in addition to the reporter cysteine in TMD2 (B). The gray and black circles indicate mutant receptors for which binding activities were slightly reduced (gray) or significantly reduced (black) after the treatment with MTSEA. White circles indicate those mutant receptors that were insensitive to MTSEA treatment or positions that resulted in little or no detectable binding when substituted for cysteine.

Our study showed that position 2.50, a well conserved aspartic acid residue found in many class A receptors, appears to be facing the binding pocket in the ground state but not in the active state of the receptor, Asp-74(2.50)-N111G-AT1, and A89C(2.65)-N111G-AT1 against the alkylating effect of MTSEA, suggesting that these residues are located in the binding pocket (Fig. 6).

Our study showed that position 2.50, a well conserved aspartic acid residue found in many class A receptors, appears to be facing the binding pocket in the ground state but not in the N111G-AT1 background. This position in TMD2 is known to be crucial for G protein activation (6, 33, 34). Also, this residue is suggested to be a part of an intermolecular bonding network implicating interhelical interactions between TMD2 and TMD7. Indeed, the recently elucidated crystal structure of squid rhodopsin shows that the carboxyl group of Asp-90(2.50) interacts directly with the side chain of Asn-311(7.61), corresponding to a state in which rhodopsin is able to bind G pro-
The divergence in the sensitivity of Cys-substituted mutants in the wild-type background and in the N111G-AT1 receptor background suggests that the accessibility of residues in TMD2 and their spatial proximity within the binding pocket were altered due to the single substitution of an asparagine for a glycine at position 111 in TMD3. Our results point to a considerable structural change during the process of activation of the receptor. In the N111G-AT1 receptor background, Val-86(2.62), which is located near the top of TMD2, became MTSEA-sensitive. This residue is located at the extreme right of the helical face formed by the MTSEA-sensitive Thr-88(2.64), Leu-81(2.57), Ala-85(2.61), and Ala-89(2.62) residues identified in the ground state (Fig. 8). The simple explanation for such a gain in sensitivity of mutant V86C-N111G-AT1 is that upon activation, TMD2 slightly rotates clockwise, bringing Val-86(2.62) within the binding pocket, where it can be alkylated by MTSEA. If this were the case, one should expect that the sensitive residue at the extreme left of the helical face forming the binding pocket would be displaced out of the binding pocket, where it could not be alkylated by MTSEA. Because T88C(2.64), N111G-AT1, is still sensitive to MTSEA, this explanation is unlikely. A clockwise rotation of TMD2 cannot either explain the reduced sensitivity of mutant L81C(2.57)-N111G-AT1 and the complete loss of sensitivity of mutant D74C(2.50)-N111G-AT1. The straightforward explanation for the sensitivity changes observed between the mutant in the ground state and those in the constitutively active state could be that TMD2 undergoes integral pivoting, bringing the top of TMD2 toward the binding pocket and pushing the bottom of TMD2 away from the binding pocket. This simple pivoting movement would then expose Val-86(2.62) to the binding pocket and extrude Asp-74(2.50) out of the binding pocket.

This explanation would go along with the four proposed simple but varied types of movements (pivoting, rotation, translation, and piston movements) that TM α-helices can undergo in a lipid bilayer (35). Previous studies using the SCAM approach have proposed a rigid body rotation of TMD2 for the constitutively active rat AT1 receptor bearing mutations at residue Asn-111(3.15) (36). In that study (36), a decrease in the accessibility of the residues in the bottom of TMD2 to the binding pocket in the case of constitutively active receptor was observed. This corresponds partly to our observations and could be interpreted as we suggest as a pivoting movement, where the bottom of the TMD2 is pushed away from the binding pocket.

In conclusion, we show that TMD2 participates in the formation of the binding pocket of the AT1 receptor. Our data comparing the ground state versus an activated state of the AT1 receptor strongly point toward a pivoting movement of TMD2 that exposes Val-86(2.62) and alters the exposure of Leu-81(2.57) to the binding pocket. The movement of the bottom of TMD2 would shift Asp-74(2.50) away from the binding pocket. This particular movement could be a structural feature common to other rhodopsin-like GPCRs.

REFERENCES
1. de Gasparo, M., Catt, K. J., Inagami, T., Wright, J. W., and Unger, T. (2000) Pharmacol. Rev. 52, 415–472
2. Burnier, M. (2001) Circulation 103, 904–912
3. Miura, S., Saku, K., and Karnik, S. S. (2003) Hypertens. Res. 26, 937–943
4. Balla, T., Baukal, A. J., Guillemette, G., Morgan, R. O., and Catt, K. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9323–9327
5. Kojima, I., Kojima, K., Kreutter, D., and Rasmussen, H. (1984) J. Biol. Chem. 259, 14448–14457
6. Gether, U., and Kobilka, B. K. (1998) J. Biol. Chem. 273, 17979–17982
7. Ghanouni, P., Steenhuis, J. J., Harrens, D. L., and Kobilka, B. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5997–6002
8. Dunham, T. D., and Farrens, D. L. (1999) J. Biol. Chem. 274, 1683–1690
9. Inoue, Y., Nakamura, N., and Inagami, T. (1997) J. Hypertens. 15, 703–714
10. Palczewski, K., Kumasaka, T., Hori, T., et al. (2000) Science 289, 739–745
11. Boucard, A. A., Wilkes, B. C., Laporte, S. A., Escher, E., Guillemette, G., and Leduc, R. (2000) Biochemistry 39, 9662–9670
12. Laporte, S. A., Boucard, A. A., Servant, G., Guillemette, G., Leduc, R., and Escher, E. (1999) Mol. Endocrinol. 13, 578–586
13. Peredon, J., Deraet, M., Auger-Messier, M., et al. (2002) Biochemistry 41, 14348–14356
14. Joseph, M. P., Maigret, B., Bonnafous, J. C., Marie, J., and Scheraga, H. A. (1995) J. Protein Chem. 14, 381–398
15. Balmforth, A. J., Lee, A. J., Warburton, P., Donnelly, D., and Ball, S. G. (1997) J. Biol. Chem. 272, 4245–4251
16. Gröbiewski, T., Maigret, B., Larguier, R., Lombard, C., Bonnafous, J. C., and Marie, J. (1997) J. Biol. Chem. 272, 1822–1826
17. Feng, Y. H., Miura, S., Husain, A., and Karnik, S. S. (1998) Biochemistry 37, 15791–15798
18. Javitch, J. A., Li, X., Kaback, J., and Karlin, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10355–10359
19. Javitch, J. A., Shi, L., and Liapakis, G. (2002) Methods Enzymol. 343, 137–156
20. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) Science 258, 307–310
21. Martin, S. S., Boucard, A. A., Clement, M., Escher, E., Leduc, R., and Guillemette, G. (2004) J. Biol. Chem. 279, 51415–51423
22. Martin, S. S., Holleran, B. J., Escher, E., Guillemette, G., and Leduc, R. (2007) Mol. Pharmacol. 72, 182–190
23. Boucard, A. A., Roy, M., Beaulieu, M. E., Lavigne, P., Escher, E., Guillemette, G., and Leduc, R. (2003) J. Biol. Chem. 278, 36628–36636
24. Fraker, P. J., and Speck, J. C., Jr. (1978) Mol. Pharmacol. 16, 7961–7968
25. Park, J. H., Scheerer, P., Hofmann, K. P., Choe, H. W., and Ernst, O. P. (2009) Biochemistry 48, 415–424
26. Fraker, P. J., and Speck, J. C., Jr. (1978) J. Biol. Chem. 253, 11929–11935
27. Dunham, T. D., and Farrens, D. L. (1999) J. Biol. Chem. 274, 36628–36636
28. Hildebrand, R. W., Scheerer, P., Park, J. H., Choe, H. W., Piechnick, R., Ernst, O. P., Hofmann, K. P., and Heck, M. (2009) PLoS ONE 4, e4382
29. Baldwin, J. M. (1993) EMBO J. 12, 1693–1703
30. Proulx, C. D., Holleran, B. J., Boucard, A. A., Escher, E., Guillemette, G., and Leduc, R. (2008) Mol. Pharmacol. 74, 552–561
31. Matthews, E. E., Zoonens, M., and Engelman, D. M. (2006) Cell 127, 447–450
32. Miura, S., and Karnik, S. S. (2002) J. Biol. Chem. 277, 24299–24305