The Fifth Transmembrane Domain of Angiotensin II Type 1 Receptor Participates in the Formation of the Ligand-binding Pocket and Undergoes a Counterclockwise Rotation upon Receptor Activation*

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The octapeptide hormone angiotensin II 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. The role of the fifth transmembrane domain (TMD5) was investigated using the substituted cysteine accessibility method. All of the residues within Thr-190 to Leu-217 region were mutated one at a time to cysteine, and after expression in COS-7 cells, the mutant receptors were treated with the sulfhydryl-specific alkylation 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 L197C-AT1, N200C-AT1, I201C-AT1, G203C-AT1, and F204C-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 TMD5 reporter cysteines engineered in a constitutively active N111G-AT1 receptor background. Indeed, mutant I201C-N111G-AT1 became more sensitive to MTSEA, whereas mutant G203C-N111G-AT1 lost some sensitivity. Our results suggest that constitutive activation of AT1 receptor causes an apparent counterclockwise rotation of TMD5 as viewed from the extracellular side.

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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 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 (TMDs), which provide structural support for signal transduction. The AT1 receptor interacts with the G protein Gq11, 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). For the AT1 receptor, it was proposed that TMD3, TMD5, TMD6, and TMD7 participate in the activation process 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, which forces the receptor to form new interactions or sever existing interactions between the residues forming 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 be formed between its seven mostly hydrophobic transmembrane domains and would be accessible to charged water-soluble agonists, 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

*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 AT$_1$ 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 AT$_1$ 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 AT$_1$ 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 a orientation similar to that of the wild-type residue. In TMDs, the sulfhydryl of a cysteine oriented toward the binding site pocket should react more quickly with a positively charged sulfhydrl reagent like methanethiosulfonate-ethylammonium (MTSEA) than sulfhydryls facing the interior of the protein or the lipid bilayer. Hence, two criteria are used for identifying engineered cysteines on the surface of the binding site pocket: (i) the reaction with MTSEA alters ligand binding irreversibly and (ii) the reaction is retarded by the presence of ligand. We previously used this approach to identify residues in TMD2, TMD3, TMD6, and TMD7 that contribute to the binding site pocket of the wild-type AT$_1$ receptor and in the constitutively active N111G-AT$_1$ receptor (21–24). Here we report the application of SCAM to probe TMD5 in the wild-type and constitutively active receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, bacitracin, and soybean trypsin inhibitor were from Sigma. The sulfhydryl-specific alkylating reagent MTSEA (CH$_3$SO$_2$-SCH$_2$CH$_2$NH$_3$) was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). The cDNA clone for the human AT$_1$ receptor subcloned in the mammalian expression vector pcDNA3 was kindly provided by Dr. Sylvain Meloche (Universit´e de Montr´eal). LipofectAmine2000, and culture media were obtained from Invitrogen. $^{125}$I-[Sar$^1$, Ile$^8$]AngII (specific radioactivity, $\sim$1500 Ci/mmol) was prepared with Iodo-GEN (Perbio Science, Erembodegem, Belgium) according to the method of Fraker and Speck (25) and as previously reported (26).

**Numbering of Residues in TMD5**—Residues in TMD5 of the human AT$_1$ receptor were given two numbering schemes. First, residues were numbered according to their positions in the human AT$_1$ 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 (27). By definition, the most conserved residue was assigned the position index “50,” e.g. in TMD5, Pro-207 is the most conserved residue and was designated Pro-207$_{5.50}$, whereas the upstream residue was designated Phe-206$_{5.49}$ and the downstream residue, Phe-208$_{5.51}$. This indexing simplified the identification of aligned residues in different GPCRs.

**Oligodeoxynucleotides Site-directed Mutagenesis**—Site-directed mutagenesis was performed on the wild-type AT$_1$ 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 Thr-190$_{5.33}$ and Leu-217$_{5.60}$. 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 AT$_1$ sequence using MultAlin (28).

**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 x 10$^6$ cells/dish. When the cells reached $\sim$90% confluency, they were transfected with 4 ug of plasmid DNA and 15 uL of LipofectAmine2000$^\text{®}$. After 24 h, the transfected cells were trypsinized, distributed into 12-well plates, and grown for an additional 24 h in complete DMEM containing 100 uL/mI penicillin and 100 uM/ml streptomycin before the MTSEA treatment and binding assay.

**Binding Experiments**—COS-7 cells were grown for 36 h posttransfection in 100-mm culture dishes, washed once with phos- phate-buffered saline (PBS), and subjected to one freeze-thaw cycle. The broken cells were then gently scraped into washing buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl$_2$), centrifuged at 2500 x 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% bovine serum albumin, 0.01% bacitracin, 0.01% soybean trypsin inhibitor). Saturation binding experiments were done by incubating broken cells (20 – 40 ug of protein) for 1 h at room temperature with increasing concentrations of [Sar$^3$, Ile$^8$]AngII in a final volume of 500 uL. Nonspecific binding was determined in the presence of 1 uM unlabeled [Sar$^4$, Ile$^8$]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 $\gamma$ counting.

**Treatment with MTSEA**—MTSEA treatment was performed according to the procedure of Javitch et al. (29), 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 nM $^{125}$I-[Sar$^3$, Ile$^8$]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 $\gamma$ counting. The percentage of fractional binding inhibition was calculated as $[1 – (specific binding after the MTSEA treatment/specific binding without the treatment)] \times 100$.

**Protection against MTSEA Reaction by [Sar$^4$, Ile$^8$]AngII**—Transfected cells grown in 12-well plates were washed once
with PBS and incubated in the presence or absence of 100 nM [Sar\textsuperscript{1}, Ile\textsuperscript{8}]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 [Sar}^{1}, \text{Ile}^{8}]\text{AngII}] - (\text{inhibition in the presence of [Sar}^{1}, \text{Ile}^{8}]\text{AngII}) / (\text{inhibition in the absence of [Sar}^{1}, \text{Ile}^{8}]\text{AngII}) \times 100.

**Phospholipase C Assay**—Inositol phosphates accumulation was determined as described previously (30). In brief, COS-7 cells were seeded in six-well plates, transfected, and labeled for 16 h in serum-free M199 containing 10 \( \mu \text{Ci/mL [myo-}^{3}\text{H]inositol (MP Biomedicals, Solon, OH). The cells were washed twice with PBS, 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.**

The 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 × g for 30 min. The upper phase 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 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**—The results are presented as the means ± S.D. Specific binding data (\( B_{\text{max}} \) 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 TMD5**—To establish whether residues in TMD5 orient themselves toward the binding site pocket of the AT\(_{1}\) receptor, we mutated 27 consecutive residues between Thr-190\textsuperscript{(5,33)} and Leu-217\textsuperscript{(5,40)} 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 125I-[Sar\textsuperscript{1}, Ile\textsuperscript{8}]AngII such as \( K_{d} \) and \( B_{\text{max}} \) were determined (Table 1).

Most mutant AT\(_{1}\) receptors exhibited high binding affinity for 125I-[Sar\textsuperscript{1}, Ile\textsuperscript{8}]AngII (similar to that of the wild-type AT\(_{1}\) receptor) except for mutant G203C\textsuperscript{(5,46)}. AT\(_{1}\) that showed a moderate 5-fold decrease in binding affinity. The mutant receptors G196C\textsuperscript{(5,39)}-AT\(_{1}\), K199C\textsuperscript{(5,42)}-AT\(_{1}\), and P207C\textsuperscript{(5,50)}-AT\(_{1}\) did not demonstrate any detectable binding activity and were not used for the SCAM analysis. \( B_{\text{max}} \) values for all detectable receptors ranged from 0.2 to 7.9 pmol/mg of protein.

**Effect of Extracellularly Added MTSEA on the Binding Properties of Mutant Receptors**—To verify whether the reporter cysteines introduced into TMD5 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 had initially verified whether the wild-type AT\(_{1}\) receptor, which contains 10 endogenous cysteines (Fig. 1) was sensitive to MTSEA. Fig. 2 shows that various concentrations of MTSEA had very little effect (no more than a 15% reduction at high MTSEA concentrations) 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 L197C\textsuperscript{(5,40)}-AT\(_{1}\) (binding inhibition of 35%), N200C\textsuperscript{(5,43)}-AT\(_{1}\) (binding inhibition of 38%), G203C\textsuperscript{(5,46)}-AT\(_{1}\) (binding inhibition of 57%), and F204C\textsuperscript{(5,47)}-AT\(_{1}\) (binding inhibition of 30%), 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 receptor I215C\textsuperscript{(5,46)}-AT\(_{1}\) were also slightly affected (Fig. 3B). Overall, the most reactive cysteines were those substituted for L197C\textsuperscript{(5,40)}, N200C\textsuperscript{(5,43)}, G203C\textsuperscript{(5,46)} and F204C\textsuperscript{(5,47)}, whereas the cysteine substituted for I215C\textsuperscript{(5,46)} was less reactive.

**Altered Accessibility to TMD5 Reporter Cysteines in the Constitutively Active N111G-AT\(_{1}\) Receptor**—We made use of the constitutively active N111G-AT\(_{1}\) receptor to assess and map the potentially altered accessibility of MTSEA to the engineered cysteines. We first determined the pharmacological properties of the 27 cysteine-substituted mutant receptors.

**TABLE 1**

| Mutant Receptor | \( K_{d} \) nM | \( B_{\text{max}} \) pmol/mg | n |
|-----------------|---------------|-----------------|---|
| Wild type       | 0.9 ± 0.4     | 1.3 ± 0.9       | 18 |
| T190C           | 0.8 ± 0.2     | 0.6 ± 0.3       | 3  |
| L191C           | 2.2 ± 0.4     | 0.7 ± 0.4       | 3  |
| P192C           | 1.0 ± 0.3     | 0.2 ± 0.02      | 3  |
| L193C           | 2.1 ± 0.3     | 1.0 ± 0.4       | 4  |
| G194C           | 0.7 ± 0.3     | 2.1 ± 0.5       | 3  |
| L195C           | 3.5 ± 1.0     | 0.9 ± 0.4       | 3  |
| L197C           | 1.8 ± 0.2     | 0.9 ± 1.4       | 3  |
| T198C           | 0.8 ± 0.2     | 1.3 ± 1.1       | 3  |
| N200C           | 0.9 ± 0.2     | 0.7 ± 0.4       | 3  |
| I201C           | 1.0 ± 0.3     | 1.2 ± 0.4       | 3  |
| L202C           | 1.5 ± 0.3     | 1.2 ± 0.2       | 3  |
| G203C           | 5.0 ± 2.8     | 0.6 ± 0.5       | 3  |
| F204C           | 2.4 ± 0.4     | 0.8 ± 0.5       | 3  |
| L205C           | 0.9 ± 0.4     | 2.5 ± 1.5       | 4  |
| F206C           | 0.9 ± 0.3     | 4.8 ± 3.2       | 4  |
| F208C           | 1.1 ± 0.6     | 1.4 ± 0.3       | 3  |
| L209C           | 0.8 ± 0.3     | 0.6 ± 0.1       | 3  |
| I210C           | 1.2 ± 0.6     | 1.7 ± 1.0       | 3  |
| L211C           | 0.7 ± 0.2     | 2.9 ± 1.0       | 4  |
| L212C           | 0.8 ± 0.3     | 3.6 ± 0.3       | 3  |
| T213C           | 0.8 ± 0.2     | 7.0 ± 0.3       | 3  |
| S214C           | 1.1 ± 0.2     | 1.8 ± 1.9       | 3  |
| Y215C           | 2.0 ± 0.3     | 2.4 ± 1.3       | 3  |
| T216C           | 0.8 ± 0.1     | 2.1 ± 0.7       | 3  |
| L217C           | 1.3 ± 0.8     | 2.8 ± 0.3       | 3  |

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Within the N111G-AT₁ receptor background, 24 cysteine-substituted mutants conserved a high binding affinity for the competitive ligand ¹²⁵I-[Sar¹, Ile⁸]AngII, whereas one mutant G203C(5.46)-N111G-AT₁ displayed a moderate almost 8-fold decrease in binding affinity (Table 2). It is interesting to note that the mutation K199C(5.42), which abolished the binding properties of the wild-type receptor, caused only a minor decrease of binding affinity in the N111G-AT₁ receptor background. The mutant receptors G196C (5.39)-N111G-AT₁, P207C(5.50)-N111G-AT₁, and L212C(5.55)-N111G-AT₁ did not have any detectable binding activity and were not used for the SCAM analysis.

**FIGURE 1. Schematic representation of the human AT₁ 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 TMD5 residues are located between Thr-190 and Leu-217 inclusively. Potential N-glycosylation sites (Asn-4, Asn-176, and Asn-188) are indicated. Asn-111 in TMD3 is also shown in gray.

**FIGURE 2. MTSEA treatment of the wild-type AT₁ receptor and sensitive reporter cysteine-bearing mutant receptors.** Intact COS-7 cells transiently expressing wild-type (WT), L197C, N200C, I201C, G203C, or F204C AT₁ receptors were incubated for 3 min at room temperature with increasing concentrations of freshly prepared MTSEA (0.5 to 6 mM). The intact cells were then incubated for 90 min at room temperature with 0.05 nM ¹²⁵I-[Sar¹, Ile⁸]AngII, and their binding properties were evaluated as indicated under “Experimental Procedures.” Each curve represents the mean ± S.D. of data obtained from at least three independent experiments.

Within the N111G-AT₁ receptor background, 24 cysteine-substituted mutants conserved a high binding affinity for the competitive ligand ¹²⁵I-[Sar¹, Ile⁸]AngII, whereas one mutant G203C(5.46)-N111G-AT₁ displayed a moderate almost 8-fold decrease in binding affinity (Table 2). It is interesting to note that the mutation K199C(5.42), which abolished the binding properties of the wild-type receptor, caused only a minor decrease of binding affinity in the N111G-AT₁ receptor background. The mutant receptors G196C(5.39)-N111G-AT₁, P207C(5.50)-N111G-AT₁, and L212C(5.55)-N111G-AT₁ did not have any detectable binding activity and were not used for the SCAM analysis. \( B_{\text{max}} \) values for all detectable receptors ranged from 0.2 to 1.9 pmol/mg of protein (Table 2).

Fig. 4 (see also Fig. 2) shows that, like the wild-type receptor, the N111G-AT₁ receptor was relatively insensitive to a 3-min treatment with MTSEA concentrations ranging from 0.5 to 2 mM, again indicating the relatively low contribution of the endogenous cysteines in the binding site pocket. Cysteine-substituted N111G-AT₁ receptor mutants were also treated...
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with increasing concentrations of MTSEA, and their binding properties were assessed with \[\text{[Sar}^1, \text{Ile}^8]\text{AngII}\]. Fig. 5 summarizes the effect of the MTSEA treatment on the different cysteine-substituted N111G-AT₁ receptor mutants. As observed in the wild-type background, mutants L197C(5.40)-N111G-AT₁ (binding inhibition of 27%), N200C(5.43)-N111G-AT₁ (binding inhibition of 43%), and F204C(5.47)-N111G-AT₁ (binding inhibition of 37%) were very sensitive to 0.5 mM MTSEA. It is noteworthy that mutation I201C(5.44), which conferred a low sensitivity to MTSEA in the wild-type background (binding inhibition of 32% after treatment with a high 2 mM concentration of MTSEA), conferred a higher sensitivity to MTSEA in the N111G-AT₁ background (binding inhibition of 48% after treatment with a low 0.5 mM concentration of MTSEA). Conversely, the mutation G203C(5.46) conferred a high sensitivity to MTSEA in the wild-type background, whereas it conferred only a low sensitivity to MTSEA in the N111G-AT₁ background. Finally, of interest, MTSEA treatment increased (127%) the binding activity of mutant K199C(5.42)-N111G-AT₁ (Fig. 5).

Protection against MTSEA Reaction by a Pretreatment with [Sar¹, Ile⁸]AngII—To confirm that reporter cysteines accessible to MTSEA are located within the binding pocket, receptor mutants were saturated with the competitive ligand [Sar¹, Ile⁸]AngII prior to MTSEA treatment. The cells were then washed with an acid buffer to dissociate the bound ligand, and the receptors were then assayed for binding with the radiolabeled competitive ligand. Fig. 6 shows how a preincubation with the competitive ligand [Sar¹, Ile⁸]AngII protected all mutant receptors that had exhibited changes in binding properties during MTSEA treatment (L197C(5.40)-AT₁, N200C(5.43)-AT₁, I201C(5.44)-AT₁, G203C(5.46)-AT₁, F204C(5.47)-AT₁, L197C(5.40)-N111G-AT₁, N200C(5.43)-N111G-AT₁, I201C(5.44)-N111G-AT₁, G203C(5.46)-N111G-AT₁, and F204C(5.47)-N111G-AT₁) from the inhibitory effect of MTSEA, with protection levels ranging from 60 to 95%.

Functional Properties of Wild-type and Mutant AT₁ Receptors—The functional properties of wild-type and mutant AT₁ receptors were evaluated by measuring the phospholipase C activity in transiently transfected COS-7 cells. Fig. 7 shows the relative amounts of inositol phosphates accumulated under basal conditions. The basal production of inositol phosphates in cells expressing the cysteine mutant receptor in the wild-type background were not significantly different from that produced

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**TABLE 2**

| Kd (nM) | Bmax (pmol/mg) | n |
|---------|----------------|---|
| N111G   | 0.7 ± 0.2      | 1.0 ± 0.7 | 8  |
| T190C   | 1.0 ± 0.2      | 0.3 ± 0.2 | 3  |
| L191C   | 2.4 ± 0.4      | 0.6 ± 0.1 | 3  |
| P192C   | 0.8 ± 0.3      | 0.5 ± 0.2 | 4  |
| I193C   | 3.2 ± 1.5      | 0.8 ± 0.3 | 3  |
| G194C   | 0.9 ± 0.7      | 1.3 ± 0.3 | 3  |
| L195C   | 2.0 ± 1.0      | 0.9 ± 0.2 | 3  |
| L197C   | 3.1 ± 1.9      | 0.2 ± 0.1 | 4  |
| T198C   | 0.6 ± 0.2      | 1.2 ± 0.3 | 3  |
| K199C   | 3.2 ± 0.7      | 0.7 ± 0.4 | 3  |
| N200C   | 1.8 ± 0.4      | 1.0 ± 0.3 | 3  |
| L201C   | 1.4 ± 0.6      | 0.8 ± 0.4 | 4  |
| L202C   | 1.4 ± 0.4      | 0.8 ± 0.4 | 3  |
| G203C   | 7.7 ± 5.5      | 0.3 ± 0.2 | 3  |
| F204C   | 1.7 ± 0.5      | 0.4 ± 0.1 | 4  |
| L205C   | 0.9 ± 0.3      | 1.1 ± 0.2 | 3  |
| F206C   | 1.8 ± 0.4      | 0.5 ± 0.3 | 3  |
| F208C   | 1.2 ± 0.1      | 0.3 ± 0.2 | 3  |
| L209C   | 0.7 ± 0.3      | 0.6 ± 0.2 | 3  |
| I210C   | 1.6 ± 0.8      | 1.2 ± 0.7 | 3  |
| I211C   | 0.7 ± 0.5      | 1.6 ± 0.6 | 4  |
| T213C   | 1.6 ± 0.6      | 1.9 ± 0.8 | 3  |
| S214C   | 1.3 ± 0.3      | 1.4 ± 0.7 | 3  |
| Y215C   | 3.1 ± 0.5      | 1.6 ± 0.5 | 3  |
| T216C   | 1.0 ± 0.8      | 1.3 ± 0.1 | 3  |
| L217C   | 1.1 ± 0.4      | 0.7 ± 0.5 | 3  |
by the cells expressing the wild-type AT$_1$ receptor. The basal level of inositol phosphates found in cells expressing cysteine mutant receptors in the N111G background was at least 4-fold higher than that found in cells expressing the wild-type AT$_1$ receptor, thereby confirming the constitutive activity of all receptors bearing both cysteine and N111G mutations. These results show that cysteine mutations within TMD5 do not compromise the functional integrity of either the ground state AT$_1$ receptor or the constitutively active N111G-AT$_1$ receptor.

**DISCUSSION**

The rationale of this study, which relied on SCAM analysis, was to gain an insight into the orientation of TMD5 of the AT$_1$ 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 background allowed us to measure relative changes in the position of certain residues, thus providing valuable information with which to infer a structural change underlying AT$_1$ receptor activation. It is important to mention that our systematic approach of substituting each residue within TMD5 for a cysteine did not alter the functional properties of either the wild-type AT$_1$ receptor or the constitutively active N111G-AT$_1$ 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 (24). Our approach of adding the MTSEA reagent to whole adherent cells expressing the AT$_1$ 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 are located from the middle (N200C(5.43), I201C(5.44), G203C(5.46), and F204C(5.47)) to the top (L197C(5.40)) portion of TMD5 (Fig. 8). These results suggest that this portion of TMD5 is involved in the interaction with the ligand. Residue Leu-197(5.40) would delineate the top, whereas residue Phe-204(5.47) would delineate the bottom of the water-accessible binding pocket of the AT$_1$ receptor. Along with these two residues, three other residues, Asn-200(5.43), Ile-201(5.44), and Gly-203(5.46), would also be part of the binding pocket in the ground state of the receptor. Indeed, by a mechanism that could be steric, electrostatic, or indirect, the alkylation of these residues with MTSEA hampered the binding of the ligand. This is further supported by the fact that the compet-
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Our finding that residues Leu-197(5.40), Asn-200(5.43), Ile-201(5.44), Gly-203(5.46), and Phe-204(5.47) are located in the binding pocket of the AT₁ receptor is in accordance with the structures of bovine rhodopsin (10), squid rhodopsin (31), opsin (32), β₁-adrenergic receptor (33), β₂-adrenergic receptor (34–36), and dopamine D2 receptor (19). Indeed, residues His-211(5.46) and Phe-212(5.47) are thought to constitute a contact point for retinal in the crystal structure of bovine rhodopsin (10), whereas residues Phe-205(5.43) and Phe-209(5.47) are located in the binding pocket of the crystal structure of squid rhodopsin (31). The crystal structure of the β₁-adrenergic receptor revealed that residues Ser-212(5.43) and Ser-215(5.46) are contact points for the high affinity antagonist cyanopindolol (33). Also, the crystal structure of the β₂-adrenergic receptor revealed that residues Ser-204(5.43), Ser-207(5.46) and Phe-208(5.47) are important for catecholamine binding (34, 35). Furthermore, using the SCAM approach, it was shown that residues Val-191(5.40), Ser-194(5.43), Ile-195(5.44), Ser-197(5.46), and Phe-198(5.47) are located in the binding pocket of dopamine D2 receptor (19). Moreover, using the methionine proximity approach, we recently observed that residue Asn-200(5.43) reacts with the photosensitive ligand [Sar¹, Bpa³]AngII (37). In light of these results, this orientation of TMD5 in the ligand-binding pocket appears to be a common feature of many class A GPCRs.

To further investigate the mechanism by which the AT₁ receptor undergoes structural changes during the transition from its inactive to its active state, we took advantage of the constitutively active N111G-AT₁ receptor. It is believed that the isomerization of conformers toward the active state, which involves transmembrane movement, is stabilized by the binding of an agonist and would be mimicked at least in part by the constitutively active receptor (6, 38). Thus, within the structural background of the N111G-AT₁ receptor, we verified the accessibility of TMD5 residues to MTSEA, and we compared the pattern obtained with that of the wild-type receptor. We found that Cys-substituted mutants L197C(5.40)-N111G-AT₁, N200C(5.43)-N111G-AT₁, and F204C(5.47)-N111G-AT₁ maintained their sensitivity to MTSEA in the constitutively active receptor background, whereas mutant I201C(5.44)-N111G-AT₁ gained sensitivity, and mutant G203C(5.46)-N111G-AT₁ lost some sensitivity to MTSEA (Fig. 5). In the protection assay, the competitive ligand [Sar¹, Ile⁸]AngII offered effective protection to all sensitive mutants (L197C(5.40)-N111G-AT₁, N200C(5.43)-N111G-AT₁, I201C(5.44)-N111G-AT₁, and F204C(5.47)-N111G-AT₁) against the alkylating effect of MTSEA, confirming that these residues are located in the binding pocket (Fig. 6).

Our finding that residues Leu-197(5.40), Asn-200(5.43), Ile-201(5.44), Gly-203(5.46), and Phe-204(5.47) face the binding pocket in the active state of the AT₁ receptor is in accordance with the current model proposed for the crystal structure of opsin (32). Indeed, residues Ile-205(5.40), Phe-208(5.43), and Phe-212(5.47) confine the binding pocket of the retinal in the active form of opsin (32, 39). In the crystal structure of activated rhodopsin (40), residues Met-207(5.43) and His-211(5.46) were also found in the binding pocket. Furthermore, using NMR spectroscopy measurements, it was found that residues His-211(5.46) and Phe-212(5.47) interact with retinal in the active intermediate form of the receptor, metarhodopsin II (41).
its interaction with [Sar¹, Ile⁸]AngII. A likely explanation is that the positive charge added by MTSEA at this position would promote ligand binding, possibly by restoring the initial charge lost by substitution of this residue for cysteine. Interestingly, when tested in the wild-type background, this mutant did not demonstrate any detectable affinity, whereas in the constitutively active background K199C(5.42)-N111G-AT₁ displayed a 3-fold decrease in binding affinity. These results suggest that alkylation of Lys-199(5.42) potentiates binding via a mechanism involving an intramolecular ionic interaction within the receptor. Further studies are needed to clarify this issue.

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 TMD5 and their spatial proximity within the binding pocket were altered because of the single substitution of an asparagine for a glycine at position 111 in TMD3. Interestingly, mutant Gly-203(5.46)-N111G-AT₁ was much less sensitive to MTSEA, whereas mutant Ile-201(5.44)-N111G-AT₁ was more sensitive to MTSEA when compared with the analogous mutants in the ground state of the receptor (see Fig. 3 versus Fig. 5). These results point to an appreciable structural change during the process of receptor activation. In fact, residue Ile-201(5.44) is located at the extreme right, whereas residue Gly-203(5.46) is located at the extreme left of the helical face formed by the MTSEA-sensitive residues Leu-197(5.40), Asp-200(5.43), and Phe-204(5.47) identified in the ground state receptor (Fig. 8). In other words, this structural change brings Ile-201(5.44) within the binding pocket where it can be more efficiently alkylated by MTSEA, whereas it pushes Gly-203(5.46) away from the binding pocket. A simple and straightforward explanation for such changes in sensitivity is that upon activation, the surface of TMD5 that is exposed to the binding site slightly rotates counterclockwise as viewed from the extracellular side. Because the MTSEA-sensitive residues lie in the upper portion of TMD5, our data do not allow us to ascribe this rotation to the entire TMD. Such a rotation 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 within a lipid bilayer (42).

It was suggested that an interaction between the highly conserved E/DRY motif on TMD3 (positions 3.49/3.50 and a glutamate residue on TMD6 (position 6.30), termed the ionic lock, stabilizes the inactive conformation on many class A GPCRs (43). Using an NMR spectroscopy approach, it was recently shown that upon activation of rhodopsin, TMD5 undergoes an apparent rotation movement (44, 45). In this process His-211(5.46) moves closer to Glu-122(3.37) of TMD3, thus disrupting the ionic lock (44, 45). Therefore the rotation of TMD5 is believed to play an important role in stabilizing the active state of the receptor.

In conclusion, we identified specific residues in the upper portion of TMD5 that participate in the formation of the ligand-binding pocket of the AT₁ receptor. Our data comparing the ground state versus an activated state of the AT₁ receptor strongly point toward a counterclockwise rotation as viewed from the extracellular side of TMD5 in which the residue facing the extreme right of the binding pocket (Ile-201(5.44)) becomes more sensitive, whereas the residue facing the extreme left of the binding pocket (Gly-203(5.46)) becomes less sensitive to the alkylating effect of MTSEA. Our results also suggest that the
binding pocket of the AT₁ receptor shares some similarities with that of other class A GPCRs.

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