Activation Induces Structural Changes in the Liganded Angiotensin II Type 1 Receptor*1

Received for publication, April 27, 2009, and in revised form, July 17, 2009 Published, JBC Papers in Press, July 27, 2009, DOI 10.1074/jbc.M109.012922

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The octapeptide hormone angiotensin II (AngII) binds to and activates the human angiotensin II type 1 receptor (hAT1) of the G protein-coupled receptor class A family. Several activation mechanisms have been proposed for this family, but they have not yet been experimentally validated. We previously used the methionine proximity assay to show that 11 residues in transmembrane domain (TMD) III, VI, and VII of the hAT1 receptor reside in close proximity to the C-terminal residue of AngII. With the exception of a single change in TMD VI, the same contacts are present on N111G-hAT1, a constitutively active mutant; this N111G-hAT1 is a model for the active form of the receptor. In this study, two series of 53 individual methionine mutations were constructed in TMD I, II, IV, and V on both receptor forms. The mutants were photolabeled with a neutral antagonist, 125I-[Sar1, p-benzoyl-L-Phe6]AngII, and the resulting complexes were digested with cyanogen bromide. Although no new contacts were found for the hAT1 mutants, two were found in the constitutively active mutants, Phe-77 in TMD II and Asn-200 in TMD V. To our knowledge, this is the first time that neuronal activation to cardiovascular cell growth and proliferation (1). Most of the known physiological effects of AngII are produced through the activation of hAT1, which belongs to the class A rhodopsin-like family of the heptahelical G protein-coupled receptor (GPCR) (2, 3). This receptor is also the target of antihypertensive therapy through antagonistic or inverse-agonistic non-peptide ligand blocking.

Like other GPCRs, the hAT1 receptor undergoes spontaneous isomerization between its inactive states (favored in the absence of an agonist) and its active state (induced or stabilized by the agonist) (4). The movement of TMD through translational or rotational displacement is believed to be essential for attaining the active state (5–7). It has been proposed that TMD III, TMD V, TMD VI, and TMD VII of the hAT1 receptor participate in the activation process by providing a network of interactions around the AngII binding pocket (8). Changes in this network are thought to be modified by agonist binding. Most of these hypotheses are, however, based on data from mutagenesis studies combined with various in silico methods. Very few investigations have used direct biochemical methods.

Such direct evidence can be collected using a photoaffinity labeling approach with receptor-reactive probes (9–11). Peptide labels containing the photoreactive amino acid p'-benzoyl-L-phenylalanine (Bpa) (Fig. 1) preferentially bond to Met residues because of their strong photochemical selectivity for thioether groups (12, 13). This selectivity has been exploited in the methionine proximity assay (MPA) where Met residues are introduced into target structures as bait to identify receptor residues in close proximity to the labeling position of the ligand (14, 15) and thus to determine the immediate molecular environment of the receptor in the vicinity of the labeling position. We used this strategy to investigate the binding environment of the hAT1 receptor to the C-terminal position of AngII (15). 125I-[Sar1,Bpa4]AngII, a neutral antagonist to the hAT1 receptor with nanomolar affinity, was used as the labeling peptide. An iterative X → Met mutagenesis walk was initially carried out on the anticipated binding pocket composed of TMD III, VI, and VII of the hAT1 receptor. We identified 11 contact residues and confirmed a deep ligand binding pocket within the transmembrane part of the receptor core, with TMD III, VI, and VII contributing to the immediate binding environment (15).

Ligand-free receptor activity can be present spontaneously or can be induced by mutations in the receptor. This concept of constitutive receptor activity was an important breakthrough in our understanding of how GPCRs can be activated (16). Constitutively active mutants (CAM) often contain point mutations that destabilize the inactive basal structure of a receptor, result-
ing in the agonist-independent activation of receptor signaling pathways (17). Substituting Asn-111 for Gly confers constitutive activity on the hAT_1 receptor (18–20), and inositol phosphate (IP) production by this mutant is similar to AngII-activated hAT_1. As such, this CAM can be used as a model of the receptor in a partially active state. Also, because [Sar^1,Bpa^8]AngII is a neutral antagonist, binding should not influence the conformation of the N111G receptor (21). An MPA study on TMDs III, VI, and VII in the N111G-hAT_1 background revealed that the contacts were the same as in the inactive state of the receptor, with the exception of T260M in the extracellular portion of TMD VI, which was no longer a contact (21). It does not seem plausible that the transition from an inactive conformation to an active state can be the result of a single, subtle change in ligand contact. We suspected that other TMD elements participate in the hAT_1 activation process. In this study we applied the MPA method to explore the remaining four TMDs (I, II, IV, and V) in the hAT_1 (basal state) and N111G-hAT_1 (active state) backgrounds to identify other ligand contacts with the C-terminal position of AngII. Such ligand contact changes between the two states were then used to infer structural changes during receptor activation of the hAT_1 receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bacitracin, bovine serum albumin, cyanogen bromide (CNBr), soybean trypsin inhibitor, and AngII were from Sigma. Culture media were from Invitrogen. [Sar^1,Ile^8]-Angiotensin II was obtained from Peptides International, Inc. (Louisville, KY). FuGENE 6 transfection reagent and Protease Inhibitor Mixture® were purchased from Roche Diagnostics. Acetonitrile was from Fisher. X-ray films (Kodak Biomax® MS, Rochester, NY) with intensifying screens from Fisher were used to visualize CNBr digestion fragments.

**Numbering of Residues**—The residues of the hAT_1 receptor were given two numbering schemes. First, residues were numbered based on their positions in the hAT_1 receptor sequence. Second, they were indexed based on their position relative to the most conserved residue in the TMD in which they are located (22). By definition, the most conserved residue was assigned the index position .50, with incremental numbering of downstream residues and decremental numbering of upstream residues.

**Oligodeoxynucleotide Site-directed Mutagenesis**—Site-directed mutagenesis was performed on the WT-hAT_1 and N111G-hAT_1 receptors using the overlap PCR method described elsewhere (23). Mutant receptors were subcloned into HindIII-XbaI sites of the mammalian expression vector pcDNA3.1. Site-directed mutations were confirmed by automated DNA sequencing.

**Synthesis and Radioiodination of Photoligands**—[Sar^1,Bpa^8]-AngII was prepared as described previously (24). 125I-AngII peptides (~1,500 Ci/mmol) were prepared using IODO-GEN® (Perbio Science, Erembodegem, Belgium) as described elsewhere (25), except that acetic acid buffer, pH 5.4, was used. The radiolabeled peptides were purified by high pressure liquid chromatography on a C-18 column (Waters) with a 20–40% (v/v) acetonitrile gradient in 0.05% aqueous trifluoroacetic acid. The specific radioactivity of the radiolabeled peptides was determined by self-displacement and saturation binding analysis.

**Cell Cultures and Transfections**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin. The cells were incubated at 37 °C in a 5% CO_2 atmosphere. Cells were transfected at ~70% confluence with FuGENE 6 transfection reagent as per the manufacturer’s instructions. Thirty six hours after the initiation of transfection, the cells were washed once with PBS (137 mM NaCl, 8.7 mM Na_2HPO_4, 3.5 mM NaHPO_4, 3.5 mM KCl, 0.9 mM CaCl_2, and 0.9 mM MgCl_2) and were stored at ~80 °C until used.

**Cell Suspensions**—Frozen transfected COS-7 cells were thawed for 1 min at 37 °C. The broken cells were then gently scraped, resuspended in 10 ml of washing buffer (100 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 5 mM MgCl_2), and centrifuged (500 × g for 10 min at 4 °C). The pellet was dispersed in binding buffer (100 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM MgCl_2, and 0.1% (w/v) bovine serum albumin).

**Binding Studies**—The broken cell suspensions (50–80 μg of protein) were incubated for 60 min at room temperature in the presence of 0.01 nM 125I-[Sar^1,Ile^8]AngII (1,500 Ci/mmol) with increasing concentrations of test peptide (15 concentrations in duplicate from 10^{-12} to 10^{-5} M, with half-log increases). Bound radioactivity was separated from free ligand by filtration at 4 °C through GF/C filters (VWR International, Inc., Montreal, QC, Canada) pre-soaked in binding buffer. Receptor-bound radioactivity was evaluated by γ-counting. Results are presented as means ± S.D. Binding data were analyzed with Prism version 5.0 for Windows (GraphPad Software, San Diego), using a one-site binding nonlinear regression analysis.

**Photoaffinity Labeling**—The broken cell suspensions (1 mg of protein) were incubated for 90 min at room temperature in the presence of 3 nM 125I-[Sar^1,Bpa^8]AngII and then centrifuged at 500 × g. The pelleted broken cells were washed once and resuspended in 0.5 ml of ice-cold washing buffer and then irradiated for 60 min on thawing ice under filtered (Raymaster black light filters number 5873, Gates and Co. Inc., Franklin Square, NY) UV light (365 nm) (100-watt mercury vapor lamp, serial number JC-Par-38, Westinghouse, Pittsburgh, PA). The suspensions were centrifuged (2,500 × g for 10 min at 4 °C), and the pellets were solubilized for 30 min at 4 °C in m-Ripa buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, and 1% (v/v) Nonidet P-40) supplemented with a protease inhibitor mixture (Complete EDTA-free) (Roche
Diagonistics. The cell lysates were centrifuged (15,000 × g for 25 min at 4 °C) to remove insoluble material, and the supernatants were stored at −20 °C until used.

Partial Purification of the Labeled Complexes—The solubilized photolabeled receptor complexes were diluted in an equal volume of 2× Laemmli buffer (200 mM dithiothreitol, 20% (v/v) glycerol, 120 mM Tris-HCl, pH 6.8, 4% (v/v) SDS, and 0.05% (w/v) bromphenol blue) and incubated for 60 min at 37 °C. SDS-PAGE was performed as described previously (26) using a 7.5% preparative gel. The gel was then cut into slices, and the radioactive content of the slices was measured by γ-counting. The labeled receptor complexes were electroeluted from the gel slices into fresh electrophoresis buffer (250 mM glycine, 25 mM Trizma base, pH 8.3, and 0.1% (w/v) SDS). The eluates (∼5 ml) were concentrated to a final volume of 0.100–0.250 ml using Amicon-10 filters (Millipore) and were stored at −20 °C until used.

CNBr Hydrolysis—The partially purified photolabeled receptors (3,500 cpm) were diluted in a 3:5 mixture of 30% (v/v) trifluoroacetic acid and CNBr dissolved in 100% acetonitrile to obtain a final concentration of 50 mg/ml. The samples were incubated at room temperature in the dark for 16–18 h. Water (1 ml) was added to terminate the reaction. The samples were lyophilized and resuspended in Laemmli buffer (200 mM dithiothreitol, 20% (v/v) glycerol, 120 mM Tris-HCl, pH 6.8, 4% (v/v) SDS). The eluates (∼5 ml) were concentrated to a final volume of 0.100–0.250 ml using Amicon-10 filters (Millipore) and were stored at −20 °C until used.

Inositol Phosphate Production—COS-7 cells were seeded in 6-well plates, transfected, and labeled for 24 h in serum-free, inositol-free Dulbecco’s modified Eagle’s medium containing 10 μCi/ml myo-[3H]inositol (Amersham Biosciences). The cells were washed twice with PBS containing 0.1% (w/v) dextrose and then incubated in stimulation buffer (Dulbecco’s modified Eagle’s medium containing 25 mM Hepes, pH 7.4, 10 mM LiCl, and 0.1% (w/v) bovine serum albumin) for 30 min at 37 °C. IP production was tested with or without 100 nM [Sar1,Bpa8]AngII, a neutral antagonist, for 10 min at 37 °C in stimulation buffer. Incubations were terminated by the addition of ice-cold perchloric acid (5% (v/v) final concentration). Water-soluble IPs 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 2,500 × g for 30 min. The upper phase containing the IPs was applied to an AG1-X8 resin column (Bio-Rad). The IPs were eluted sequentially using ammonium formate/formic acid solutions of increasing ionic strength. Fractions containing IP were collected, and radiolabeling was measured by scintillation counting.

Molecular Modeling—Calculations were performed on a Silicon Graphics Octane2 workstation (Silicon Graphics Inc., Mountain View, CA). Molecular modeling of the hAT1 receptor and the [Sar1,Bpa8]AngII-hAT1 receptor complex was performed using the INSIGHTII suite of programs (Homology, Discover, and Biopolymer, Accelrys, San Diego). The molecular model of hAT1 (GenBank™ accession number. P30556) was based on the crystal structure of rhodopsin (Protein Data Bank accession numbers 1u19). The strictly conserved residues of the hAT1 receptor and rhodopsin were aligned to identify and assign the structurally conserved regions (see supplemental material). There were no gaps in any of the TMDs. The coordinates of the structurally conserved regions were then transferred to the sequence of hAT1. Nonstructurally conserved loop regions were constructed using the generate command in the homology module. The procedure developed by Levinthal and co-workers (27) generates loop conformations with minimal potential energies by iteratively sampling the ϕ and ψ space. The potential energies of the models were minimized using Discover with Amber force field (28). The heavy atoms were fixed first, then the backbone alone, and finally the backbones of the TMDs alone. The disulfide bonds (between extracellular loops 1 and 2 and between the N-terminal and extracellular loop 3) were then added to hAT1, and the potential energy was minimized, again by fixing all the heavy atoms of the loops involved. The heavy atoms of the N-terminal were left unrestrained.

Generation of Liganded Receptor Structures by Molecular Modeling—The [Sar1,Bpa8]AngII ligand was constructed using INSIGHTII BUILDER. The Bpa molecule was placed between TMD III, VI, and VII as suggested by previous studies (15, 21) and the present photolabeling results. It was positioned to accommodate, as much as possible, the restraints used for the minimization without interfering with receptor side chains. To make this possible, the [Sar1,Bpa8]AngII ligand was made fully linear, and the Bpa moiety bound to position 8 of AngII was rotated. A first minimization of the complex between hAT1 and [Sar1,Bpa8]AngII (with the coulombic terms turned off) was performed using restraints (2.0 Å < d < 7.5 Å) between the C-β atoms of the photolabeled Met residues and the ketone oxygen of Bpa as described elsewhere (15). The minimization procedure contained many steps. First, all the heavy atoms of both hAT1 and the ligand were fixed. Then constraints on the loops and the ligand were removed. In the final step, only the backbone atoms of the TMDs were fixed. The complex was then minimized until the maximum derivative was less than 0.1 kcal/mol.

Generation of the Liganded Constitutively Active N111G Mutant Receptor—The complex between the [Sar1,Bpa8]AngII ligand and the constitutively active N111G mutant receptor was modeled by mutating the Asn-111 residue on the basal model to a glycine, adding distance restraints (2.0 Å < d < 7.5 Å) between the ketone oxygen of Bpa and the C-β atoms of the photolabeled residues and using a simulated annealing protocol for a short run molecular dynamics. During the simulation, no atoms were fixed, but the ϕ and ψ angles of all TMDs were restrained to preserve the helical structure. The simulated annealing protocol is described elsewhere (29).

RESULTS

Site-directed Mutagenesis and Binding Properties of Mutant Receptors of the Basal Form of hAT1—To identify the receptor residues that contact the C-terminal amino acid of AngII and that thus participate in the formation of the hAT1 ligand binding pocket, 53 residues in TMD I, II, IV, and V of WT-hAT1 were mutated, one at a time, to methionine (Fig. 2). Each
mutant receptor was transiently expressed in COS-7 cells. To assess the conservation of the functional integrity of these receptors following the mutations, pharmacological parameters describing the equilibrium binding of [Sar1,Bpa8]AngII such as $K_D$ and $B_{\text{max}}$ were determined (Table 1). Most mutant hAT$_1$ receptors exhibited high binding affinity for [Sar1,Bpa8]AngII similar to that of the WT-hAT$_1$ receptor, with $B_{\text{max}}$ values ranging from 456 to 1,170 fmol/mg. Some mutant receptors displayed 1,000-fold lower affinity than the wild-type $K_D$ (Y35M(1.39), A156M(4.53), A159M(4.56), G203M(5.46), and F204M(5.47)) or had no detectable binding activity (G42M(1.46) and N46M(1.50)) and were thus not used for the MPA analysis.

Binding Properties of the Met-substituted N111G-hAT$_1$ Receptor Mutants—The same 53 methionine-substituted receptor mutants were constructed in the N111G-hAT$_1$ receptor background. For the most part, these mutants also retained nanomolar binding affinities toward [Sar1,Bpa8]AngII. The $B_{\text{max}}$ values for all detectable receptors ranged from 423 to 1,171 fmol/mg (Table 2). Of these double mutant receptors, seven demonstrated a >1,000-fold decrease in affinity (V50M/N111G(1.54), N69M(2.45), D74M(2.50), and F206M(5.49)) or had no detectable binding activity (G42M/N111G(1.46) and N46M(1.50)) and were thus not used for the MPA analysis.

Photoaffinity Labeling of the hAT$_1$ and N111G-hAT$_1$ Receptor Series—To investigate the binding environment of the C-terminal residue of AngII within the hAT$_1$ and N111G-hAT$_1$ receptors, the 46 methionine mutant receptors from the hAT$_1$ series and the 44 methionine mutant receptors from the N111G-hAT$_1$ series were photolabeled with 3 nM of 125I-[Sar1,Bpa8]AngII. Fig. 3 shows the results of a typical photolabeling experiment using WT-hAT$_1$, N111G-hAT$_1$, and two selected mutants (F77M(2.53) and F77M/N111G(2.53)). All the mutants produced a broad band that migrated diffusely between 75 and 180 kDa on 7.5% SDS-polyacrylamide gels (Fig. 3, lanes 1–4). Labeling was completely prevented when the experiments were carried out in the presence of 10 μM AngII (Fig. 3, lanes 5–8).

Digestion of the Labeled Receptor Mutants—To identify the covalently modified regions of hAT$_1$ and the mutants, the labeled receptors were partially purified, treated with CNBr, and analyzed by SDS-PAGE. As observed previously for both
TABLE 1
Binding properties of |Sar1|Bpa8|AngII to methionine-substituted hAT1 mutant receptors

| Receptor | KD (nM) | Bmax (fmol/mg) | n |
|----------|---------|---------------|---|
| WT       | 0.8 ± 0.2 | 1121 ± 137 | 25 |
| Y35M1(49) | 5280 ± 200 | 945 ± 107 | 4 |
| S36M(4-40) | 1.2 ± 0.1 | 935 ± 108 | 3 |
| I27M(4-41) | 1.5 ± 0.1 | 942 ± 87 | 3 |
| I38M(4-42) | 1.4 ± 0.2 | 793 ± 68 | 3 |
| F39M(4-43) | 1.1 ± 0.2 | 646 ± 74 | 3 |
| V40M(4-44) | 1.8 ± 0.2 | 847 ± 63 | 4 |
| V41M(4-45) | 1.8 ± 0.2 | 650 ± 75 | 5 |
| F42M(4-46) | 1.3 ± 0.1 | 693 ± 64 | 5 |
| F43M(4-47) | 1.2 ± 0.1 | 1064 ± 166 | 3 |
| G45M(4-48) | 1.7 ± 0.2 | 979 ± 93 | 3 |
| S47M(4-49) | 1.4 ± 0.1 | 954 ± 134 | 4 |
| L48M(4-50) | 1.1 ± 0.2 | 463 ± 75 | 5 |
| V49M(4-51) | 1.1 ± 0.1 | 641 ± 79 | 5 |
| V50M(4-52) | 1.2 ± 0.1 | 1080 ± 116 | 5 |
| L68M(4-53) | 1.6 ± 0.1 | 933 ± 72 | 5 |
| N69M(4-54) | 8.1 ± 0.4 | 1140 ± 79 | 5 |
| L70M(4-55) | 3.2 ± 0.2 | 1120 ± 124 | 5 |
| A71M(4-56) | 1.8 ± 0.1 | 451 ± 45 | 5 |
| L72M(4-57) | 6.9 ± 0.3 | 920 ± 84 | 5 |
| A73M(4-58) | 10.1 ± 0.3 | 1062 ± 74 | 5 |
| D74M(4-59) | 1.0 ± 0.1 | 565 ± 54 | 3 |
| L75M(4-60) | 1.9 ± 0.1 | 1054 ± 71 | 3 |
| C76M(4-61) | 0.9 ± 0.1 | 645 ± 39 | 3 |
| F77M(4-62) | 1.0 ± 0.1 | 850 ± 33 | 4 |
| L83M(4-63) | 1.6 ± 0.1 | 888 ± 106 | 5 |
| N96M(4-64) | 1.2 ± 0.2 | 700 ± 114 | 3 |
| T80M(4-65) | 0.7 ± 0.1 | 606 ± 78 | 5 |
| L81M(4-66) | 1.6 ± 0.2 | 853 ± 59 | 3 |
| L83M(4-67) | 0.9 ± 0.1 | 480 ± 52 | 3 |
| L150M(4-68) | 1.4 ± 0.1 | 816 ± 93 | 4 |
| I151M(4-69) | 0.8 ± 0.1 | 687 ± 108 | 3 |
| I152M(4-70) | 0.8 ± 0.1 | 640 ± 128 | 5 |
| V153M(4-71) | 3.6 ± 0.3 | 1170 ± 131 | 3 |
| L154M(4-72) | 1.4 ± 0.4 | 631 ± 106 | 4 |
| L155M(4-73) | 1.4 ± 0.1 | 977 ± 98 | 3 |
| A156M(4-74) | 4130 ± 176 | 669 ± 78 | 5 |
| G157M(4-75) | 1.6 ± 0.2 | 1135 ± 94 | 4 |
| L158M(4-76) | 1.0 ± 0.1 | 802 ± 86 | 5 |
| A159M(4-77) | 1642 ± 77 | 524 ± 79 | 3 |
| K199M(5-43) | 4.7 ± 0.2 | 609 ± 42 | 3 |
| N200M(5-44) | 2.8 ± 0.2 | 1107 ± 97 | 5 |
| I201M(5-45) | 2.7 ± 0.2 | 1026 ± 77 | 6 |
| L202M(5-46) | 1.1 ± 0.1 | 708 ± 91 | 6 |
| G203M | 1890 ± 45 | 787 ± 119 | 7 |
| F204M(5-47) | 332 ± 104 | 622 ± 125 | 7 |
| L205M(5-48) | 1.0 ± 0.1 | 630 ± 60 | 5 |
| F206M(5-49) | 0.9 ± 0.1 | 859 ± 75 | 6 |
| F208M(5-50) | 0.9 ± 0.2 | 1023 ± 101 | 6 |
| L210M(5-51) | 1.1 ± 0.2 | 998 ± 115 | 7 |
| I210M(5-52) | 1.1 ± 0.2 | 456 ± 45 | 6 |
| I211M(5-53) | 0.9 ± 0.2 | 598 ± 78 | 7 |

Two major differences were observed among the labeled methionine mutants of the CAM series. Digestion of labeled TMD II mutant F77M/N111G1(2-53) produced a new fragment in addition to those associated with TMD VII labeling (Fig. 5, lane 4). This band co-migrated with the free ligand (Figs. 4–6, last lane), indicating ligand release. An identical pattern was also observed for the TMD V mutant N111G/N200M1(5-43) (Fig. 6, lane 3). Ligand release after CNBr digestion is the consequence of Met labeling, indicating direct contact between the ligand and the mutated position (15).

Functional Properties of Mutant Receptors—The functional properties of WT-hAT1, CAM-hAT1, and selected mutants from each series were evaluated by assessing their basal inositol

hAT1 and N111G-hAT1 (15, 21), the digested receptors produced a 7.2-kDa band corresponding to TMD VII, a band corresponding to the C-terminal sequence (285–334 plus ligand), and the typical 10.0-kDa band from incomplete digestion at position Met-334 (285–359 plus ligand) (Figs. 4–6) (30, 31). Most receptor mutants produced a receptor fragmentation pattern compatible with exclusive labeling on TMD VII, indicating that those mutated residues were not contributing to the immediate surroundings of the labeling moiety. In those cases only the characteristic 7.2-kDa (285–334) and 10.0-kDa (285–359) receptor fragments were produced. This property was observed for all mutants on the WT background, as shown for some selected mutants (Fig. 4).
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phosphate (IP) production in transiently transfected COS-7 cells. IP production by mutants in the WT background was similar to that of WT in transfected cells, whereas all mutants in the CAM background displayed constitutive activity similar to that of the N111G-hAT₁ receptor. Fig. 7 shows as examples the IP productions of MPA-positive mutants F77M/N111G-hAT₁ and TMD I mutant-hAT₁ receptors. Radiolabeled fragments are indicated as thick gray bars, and the photolabeling peptide as a thinner gray bar. The calculated molecular masses of the proteins are indicated in kDa and include the mass of the labeling peptide. The respective Met positions are indicated by bold numbers. The results are representative of experiments performed at least in triplicate.

DISCUSSION

Many benzophenone labeling studies have identified Met residues as covalent attachment sites because of the pronounced photochemical selectivity of benzophenone photolabels for Met residue (12, 13). In contrast, 125I-[Sar¹, Bpa⁸]AngII incorporates into the hAT₁ receptor at positions Phe-293(7.44) and Asn-294(7.45) of TMD VII (21, 30), strongly suggesting that no endogenous Met residues are accessible to position 8 of AngII in the hAT₁ binding pocket. Met residues introduced into the hAT₁ receptor by site-directed mutagenesis in a position proximal to the photoreactive moiety of 125I-[Sar¹, Bpa⁸]AngII should pull toward it part or all of the labeling peptide as a thinner gray bar. The calculated molecular masses of the proteins are indicated in kDa and include the mass of the labeling peptide. The respective Met positions are indicated by bold numbers. The results are representative of experiments performed at least in triplicate.

cases a neutral antagonist without influence on the basal activity of the receptor and its mutants.

FIGURE 4. A, CNBr cleavage of the photolabeled WT-hAT₁, and TMD I mutant hAT₁ receptors. Partially purified receptors photolabeled with 125I-[Sar¹, Bpa⁸]AngII were incubated with 100 mg/ml CNBr. Samples (2,500–3,500 cpm/well) were separated on 16.5% SDS-PAGE (Tris-Tricine) gels, which were bated with 125I-[Sar¹, Bpa⁸]AngII as follows: WT-hAT₁ (lane 1), N111G-hAT₁ (lane 2), F77M(2.53)-hAT₁ (lane 3), and F77M/N111G(2.53)-hAT₁ (lane 4). Photochemical selectivity of benzophenone photolabeling was also performed in the presence of 10 μM AngII as follows: WT-hAT₁ (lane 5), N111G(2.53)-hAT₁ (lane 6), F77M(2.53)-hAT₁ (lane 7), and F77M/ N111G(1)-hAT₁ (lane 8). The membranes were then exposed to UV light and solubilized. CNBr digestion of the photolabeled and solubilized receptor materials were separated on 7.5% SDS-polyacrylamide gels. The apparent molecular weights of the protein standards are indicated. Shown are the following: SDS-PAGE WT-hAT₁ (lane 1), N111G-hAT₁ (lane 2), C76M/N111G(2.52)-hAT₁ (lane 3), F77M/N111G(2.53)-hAT₁ (lane 4), L78M/N111G(2.54)-hAT₁ (lane 5), L79M/N111G(2.54)-hAT₁ (lane 6), and 125I-[Sar¹, Bpa⁸]AngII (lane 7). B, schematic degradation patterns of photolabeled WT-hAT₁, N111G-hAT₁, and TMD II mutant-N111G-hAT₁ receptors. Radiolabeled fragments are indicated as thicker gray bars and the photolabeling peptide as a thinner gray bar. The calculated molecular masses of the proteins are indicated in kDa and include the mass of the labeling peptide. The respective Met positions are indicated by bold numbers. The results are representative of experiments performed at least in triplicate.

FIGURE 5. A, CNBr cleavage of the photolabeled WT-hAT₁, N111G-hAT₁, and TMD II mutant N111G-hAT₁ receptors. Separations were performed as described in Fig. 4A. Shown are the following: WT-hAT₁ (lane 1), N111G-hAT₁ (lane 2), C76M/N111G(2.52)-hAT₁ (lane 3), F77M/N111G(2.53)-hAT₁ (lane 4), L78M/N111G(2.54)-hAT₁ (lane 5), L79M/N111G(2.54)-hAT₁ (lane 6), and 125I-[Sar¹, Bpa⁸]AngII (lane 7). B, schematic degradation patterns of photolabeled WT-hAT₁, N111G-hAT₁, and TMD II mutant-N111G-hAT₁ receptors. Radiolabeled fragments are indicated as thick gray bars and the photolabeling peptide as a thinner gray bar. The calculated molecular masses of the proteins are indicated in kDa and include the mass of the labeling peptide. The respective Met positions are indicated by bold numbers. The results are representative of experiments performed at least in triplicate.

FIGURE 3. Photolabeling of WT-hAT₁, N111G-hAT₁, and the Met mutants of the hAT₁ receptor with 125I-[Sar¹, Bpa⁸]AngII. Membranes from COS-7 cells transiently transfected with different Met mutants of hAT₁ were incubated with 125I-[Sar¹, Bpa⁸]AngII as follows: WT-hAT₁ (lane 1), N111G-hAT₁ (lane 2), F77M(2.53)-hAT₁ (lane 3), and F77M/N111G(2.53)-hAT₁ (lane 4). Photolabeling was also performed in the presence of 10 μM AngII as follows: WT-hAT₁ (lane 5), N111G(2.53)-hAT₁ (lane 6), F77M(2.53)-hAT₁ (lane 7), and F77M/ N111G(1)-hAT₁ (lane 8). The membranes were then exposed to UV light and solubilized. CNBr digestion of the photolabeled and solubilized receptor materials were separated on 7.5% SDS-polyacrylamide gels. The apparent molecular weights of the protein standards are indicated. Shown are the following: SDS-PAGE WT-hAT₁ (lane 1), N111G-hAT₁ (lane 2), C76M/N111G(2.52)-hAT₁ (lane 3), F77M/N111G(2.53)-hAT₁ (lane 4), L78M/N111G(2.54)-hAT₁ (lane 5), L79M/N111G(2.54)-hAT₁ (lane 6), and 125I-[Sar¹, Bpa⁸]AngII (lane 7). B, schematic degradation patterns of photolabeled WT-hAT₁, N111G-hAT₁, and TMD II mutant-N111G-hAT₁ receptors. Radiolabeled fragments are indicated as thick gray bars and the photolabeling peptide as a thinner gray bar. The calculated molecular masses of the proteins are indicated in kDa and include the mass of the labeling peptide. The respective Met positions are indicated by bold numbers. The results are representative of experiments performed at least in triplicate.
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FIGURE 6. A, CNBr cleavage of the photolabeled WT-hAT₁, N111G-hAT₁, and TMD V mutant N111G-hAT₁ receptors. Separations were performed as described in Fig. 4A. WT-hAT₁ (lane 1), N111G-hAT₁ (lane 2), N111G/N200M (5.43)-hAT₁ (lane 3), N111G/I201M (5.46)-hAT₁ (lane 4), N111G/L202M (5.45)-hAT₁ (lane 5), N111G/G203M (5.40)-hAT₁ (lane 6), and 125I-[Sar¹,Bpa⁸]AngII (lane 7). B, schematic degradation patterns of photolabeled WT-hAT₁, N111G-hAT₁, and TMD V mutant N111G-hAT₁ receptors. Radioactively labeled fragments are indicated as thick gray bars and the photolabeling peptide as a thinner gray bar. The calculated molecular masses of the proteins are indicated in kDa and include the mass of the labeling peptide. The respective Met positions are indicated by bold numbers. The results are representative of experiments performed at least in triplicate.

FIGURE 7. Basal levels of IP in COS-7 cells expressing the WT-hAT₁ and mutant hAT₁ receptors. Transfected COS-7 cells were loaded for 16–24 h with 10 μCi/ml myo-[³H]inositol in inositol-free M199 medium. IP levels (sum of inositol bisphosphate, inositol trisphosphate, and inositol tetrakisphosphate) were determined as described under “Experimental Procedures.” The shown results are the means ± S.D. of a typical experiment carried out in triplicate (from three identical experiments). IP production was normalized via the expression levels listed in Tables 1 and 2. Basal activities are represented by open columns and IP production in presence of 100 nm [Sar¹,Bpa⁸]AngII by solid columns.

out lengthy analytical steps such as purification, protein digestion, and other sequence determination approaches.

An initial MPA study was performed on what was suspected to be the core of the AngII binding pocket (TMD III, VI, and VII of hAT₁) (15), followed by a parallel study on the CAM form of hAT₁ (N111G-hAT₁) (21). In both studies, the same 10 contacts were found, except for T260M (6.55), which was only labeled in the basal form of hAT₁ (15, 21). It is highly unlikely results are representative of experiments performed at least in triplicate.

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We constructed two series of 53 hAT₁ mutants with X → Met mutations in TMD I, II, IV, or V on the WT-hAT₁ receptor and its CAM derivative (Fig. 2). We did not identify any new ligand contacts by photolabeling the basal receptor series on hAT₁, with the photoligand 125I-[Sar¹,Bpa⁸]AngII followed by CNBr digestion of the partially purified receptors. All the labeled mutant receptors displayed the classical WT-hAT₁ profile when digested, with non-Met contacts on TMD VII being labeled (Fig. 4A). The residues of the four TMDs are thus not in close proximity or accessible to the C-terminal Bpa side chain and thus do not participate in the bimolecular ligand-receptor interface of position 8 of AngII in the hAT₁ receptor ground state. On the other hand, we observed new fragment patterns for mutants F77M/N111G (2.53) (TMD II) and N111G/N200M (5.43) (TMD V) upon CNBr digestion of the CAM receptor series (Figs. 5 and 6). These patterns showed mostly non-Met labeling of TMD VII as well as some ligand release (Figs. 5 and 6) and Met labeling. These results indicated that these two residues were closer or more accessible to the C-terminal labeling residue of the AngII analogue in the active form. The low proportion of ligand release compared with the relatively intense TMD VII labeling of the two mutants suggested that these contacts were peripherally located in the labeling sphere of Bpa reactivity. They also strongly suggested that TMD II and TMD V participate in the bimolecular ligand receptor interface and are thus part of the ligand binding domain in the active form of hAT₁. Interestingly, a substituted cysteine accessibility method (SCAM) study on TMD II of hAT₁ showed that most of the reagent-accessible residues were in the middle (D74C(2.50) and L81C(2.57)) to top portion of TMD II (A85C(2.61), T88C(2.64), and A89C(2.65)) (33). F77M(2.53) is located on the same helix face but was not identified by either MPA or SCAM in the ground state of the receptor. This SCAM study suggested that, upon activation via the N111G mutation, a pivoting movement shifts the cytoplasmic portion of TMD II D74(2.50) away from the binding pocket (33). In our MPA analysis in the N111G-hAT₁
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receptor background, such a movement would expose residue 77(2.53) to the binding pocket, allowing labeling of the C-terminal position of AngII, which is indeed observed in F77M/N111G-hAT₁. In TMD V, position Asn-200(5.43) in the N111G-hAT₁ receptor background also contacts the ligand in β₂-adrenergic, bovine rhodopsin, and squid rhodopsin crystal structures (34–36). The molecular mechanisms by which agonists bind to and activate GPCRs through conformational changes are the object of much research effort. Although for many years the only available GPCR crystal structure was the one of rhodopsin (35), the crystal structures of other GPCRs such as the β₁-adrenergic (37), β₂-adrenergic (38), ligand-free opsin (39), opsin Gs-bound (40), squid rhodopsin (36), and A2A-adenosine receptors (41) have recently been determined.

To select the GPCR structure that most closely resembles the ligand interaction of the hAT₁ receptor for homology-based molecular modeling, we compared the residues facing the binding pockets from the published crystal structures (42) with our experimentally determined contact points of hAT₁. Seven MPA contacts in hAT₁ (2.53, 3.36, 6.44, 6.48, 6.51, 6.55, and 7.45) are found in all crystal structures. However, position 3.37 does not participate in the binding pocket of either squid rhodopsin or the β₁. Furthermore, the same holds true for 5.43 in both Gs-bound opsin and free opsin, thus eliminating those four structures. Within the remaining three structures, the A₂A, the β₂, and rhodopsin, we compared the interaction proximity of their respective ligands with those nine residues. Rhodopsin corresponded most closely to the hAT₁ contacts; therefore, the rhodopsin structure was chosen as a template for homology-based modeling of both the hAT₁ (ground state) and N111G-hAT₁ (active state) receptors by the already existing wealth of experimental results on rhodopsin activation.

To assess the relative positions of the residues identified in the binding pocket of the hAT₁ receptor, we incorporated data from previous studies (15, 21) with the results described here to develop two molecular models. We generated a liganded hAT₁ structure where all experimentally determined contacts were within ±8 Å of the Bpa ketone oxygen. The first model was based on the [Sar¹,Bpa⁸]AngII-hAT₁ receptor complex and represents the receptor in the ground state (Fig. 8A). To further investigate the mechanism by which the hAT₁ receptor undergoes structural changes during the transition from its inactive to its active state, we took advantage of the constitutively active N111G-hAT₁ receptor to develop a second model, the [Sar¹,Bpa⁸]AngII-N111G receptor complex (Fig. 8B). Both complexes were modeled using the distance restraints determined in previous photolabeling studies (30, 32) to correctly position [Sar¹,Bpa⁸]AngII in the receptor binding pocket (Fig. 8). Both models showed that, for the most part, the main core of the receptor binding pocket remains unchanged because residues Leu-112(3.36), Tyr-113(3.37), Phe-249(6.44), Trp-253(6.48), His-256(6.51), and Phe-293(7.44) through Lys-297(7.48) remain...
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close to position 8 of AngII. The active state model showed that residues F77M(2.53) and N200M(5.43), which cannot be photolabeled by the ligand in the basal form of hAT1, are positioned closer to the ligand in the N111G background (Fig. 8B). We previously showed that residue T260M(6.53) interacts with the ligand in the basal form (15) but that this interaction is lost in the activated form of hAT1. This was assumed to be an outward movement of the extracellular portion of TMD VI, away from the binding pocket (21). However, the model we present here appears to contradict this interpretation, because it shows TMD VI moving toward the binding pocket (Fig. 8C). One explanation for this discrepancy is that the major structural changes observed in TMD V and VI, where the extracellular portions of both TMDs move closer to the binding pocket, could restrict the scope of action of the Bpa side chain in the portions of both TMDs moving closer to the binding pocket.

The positions of TMD I and IV appear practically unchanged as they move slightly clockwise (viewed from the extracellular portion), and TMD VII moves slightly away from the binding pocket, although TMD II moves slightly toward the binding pocket. The movements of TMD I and IV appear practically unchanged (Fig. 8C).

Several mutants displayed impaired binding properties. One group of mutants (Y35M(1.39), G203M(5.46), and F204M(5.47)) lost their binding affinity toward [Sar1,Bpa8]AngII in the basal form of hAT1. These mutants are noteworthy because the mutations are in key positions for ligand binding in other GPCRs (34–39). In the bovine rhodopsin and opsin receptors, positions 1.39, 5.46, and 5.47 face the retinal binding pocket. Positions 5.46 and 5.47 have also been reported to be in close proximity to the ligand in the β1-adrenergic, β2-adrenergic, and squid rhodopsin receptors (34, 38). A second group is composed of binding-impaired mutants where either Ala or Gly is replaced by Met (G42M(1.46), G42M/N111G(1.46), A156M(4.53), N111G/A156M(4.53), A159M(4.56), N111G/A159M(4.56), and G203M(5.46)). We hypothesized that the increased bulk of the Met side chain might lead to steric hindrance, thus impeding binding with [Sar1,Bpa8]AngII in both the hAT1 and N111G-hAT1 series. In a third group of mutants (N46M(1.50), N46M/N111G(1.50), and D74M/N111G(2.50)), it was not surprising that binding was impaired because these positions are the most conserved in these TMDs.

Rhodopsin activation mechanisms are the best studied and understood in the GPCR domain with 15 x-ray structures solved of bovine visual pigment in different activation states and their relationship to other GPCR (43). Some of the main considerations relevant to these presented results will be briefly discussed. Early electron paramagnetic resonance spectroscopy studies provided evidence that photoactivation of rhodopsin involves rotation and tilting of TMD VI relative to TMD III (44) with additional support by chemical reactivity measurements and fluorescence spectroscopy (5), as well as ultraviolet absorbance spectroscopy (45) and zinc chelation of histidine mutants (46), for motion of TMD VI. A similar movement of TMD VI has been reported with a zinc-chelating mutant of the β2-adrenergic receptor (47) during activation. Recent results on rhodopsin activation confirm an important movement of principal TMD V and TMD VI with a site-directed fluorescence labeling approach (48), as well as a Fourier-transformed infrared analysis of noncanonical p-azidophenylalanine mutated rhodopsin (49). The crystal structure of light-activated and Schiff-base deprotonated rhodopsin identifies the immediate retinal contact environment (50). 212F(5.43) contacts directly retinal in irradiated rhodopsin, as well as 6.48 and 6.51. Residue 5.43 corresponds to the N111G/N200M(5.43), one of the two MPA positive contacts in this study, and 6.48 and 6.51 are also contacts in both receptor forms. According to a solid-state NMR study on rhodopsin (51), extracellular loop II and TMD V undergo movements during activation.

Taking into account these experimental results leads to proposing a receptor activation model for the hAT1 receptor. It is proposed that, during the activation process, the extracellular portions of TMDs II, V, and VI move closer to the binding pocket and that TMD VII moves away from it. These movements would lead to conformational changes in intracellular loop 3 and the C-tail, leading to G protein activation. Concomitant SCAM studies on the hAT1 receptor also support the proposed activation mechanism (52–54). On the nonliganded receptor they indicate that the N111G mutation causes TMD VII to move away from the binding pocket, TMD III to rotate, and the extracellular portion of TMD VI to tilt inward toward the binding pocket while simultaneously distorting the bottom (intracellular side) away from the binding pocket.

In conclusion, the MPA made it possible to experimentally determine the binding environment of a given ligand residue. The determination of these ligand contacts also allowed the construction of two evidence-based models of the hAT1 receptor in its basal and N111G active states, both based on the rhodopsin structure. To our knowledge, we showed for the first time a direct ligand contact with TMD II and V, participating in the hAT1 activation process.

Acknowledgment—We are grateful to Marie-Reine Lefebvre for the expert help and technical assistance.

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