Activation of the AT₁ Angiotensin Receptor Is Dependent on Adjacent Apolar Residues in the Carboxylic Terminus of the Third Cytoplasmic Loop*  

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Meng Zhang, Xue Zhao, Hao-Chia Chen, Kevin J. Catt, and László Hunyady‡§  
From the Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892-4510 and the ³Department of Physiology, Semmelweis University Medical School, P. O. Box 259, H-1444 Budapest, Hungary

The C-terminal region of the third intracellular loop of the AT₁ angiotensin receptor (AT₁-R) is an important determinant of G protein coupling. The roles of individual residues in agonist-induced activation of Gq,11-dependent phosphoinositide hydrolysis were determined by mutational analysis of the amino acids in this region. Functional studies on mutant receptors transiently expressed in COS-7 cells showed that alanine substitutions of the amino acids in positions 232–240 of the third loop had no major effect on signal generation. However, deletion mutations that removed Ile²³⁸ or affected its position relative to transmembrane helix VI significantly impaired angiotensin II-induced inositol phosphate responses. Substitution of Ile²³⁸ with an acidic residue abolished the ability of the receptor to mediate inositol phosphate production, whereas its replacement with basic or polar residues reduced the amplitude of inositol phosphate responses. Substitutions of Phe²³⁹ with polar residues had relatively minor effects on inositol phosphate signal generation, but its replacement by aspartic acid reduced, and by positively charged residues (Lys, Arg) significantly increased, angiotensin II-induced inositol phosphate responses. The internalization kinetics of the Ile²³⁸ and Phe²³⁹ mutant receptors were impaired in parallel with the reduction in their signaling responses. These findings have identified Ile²³⁸ and Phe²³⁹ as critical residues in the C-terminal region of the third intracellular loop of the AT₁-R for receptor activation. They also suggest that an apolar amino acid corresponding to Ile²³⁸ of the AT₁-R is a general requirement for activation of other G protein-coupled receptors by their agonist ligands.

The AT₁ angiotensin receptor (AT₁-R) is a member of the GPCR superfamily and mediates the physiological actions of the octapeptide hormone, Ang II, on cardiovascular regulation and salt/water balance. The binding of Ang II to the AT₁-R initiates conformational changes that lead to activation of its cognate G protein(s), predominantly Gq,11, in numerous Ang II target tissues. The subsequent stimulation of phospholipase C causes the generation of inositol 1,4,5-trisphosphate and diacylglycerol, leading to the elevation of [Ca²⁺] and activation of protein kinase C (1–3). The AT₁-R is also coupled to other signaling pathways, including the Ras-mediated activation of mitogen-activated protein kinase and cell growth responses (4, 5).

Although GPCRs exhibit a remarkable degree of structural and functional diversity, all share a common architecture in which seven transmembrane helices are linked by alternating intracellular and extracellular loops. Agonist binding to the extracellular loops, and/or the exofacial regions of the transmembrane domains, induces conformational changes in the transmembrane helices and intracellular regions that promote coupling of the activated receptor to its cognate G protein(s). The receptor domains that are involved in G protein coupling have been analyzed in many GPCRs. Studies using chimeric α₂/β₂-, α₁β₁/α₂- and α₁β₁-β₂-adrenergic receptors and chimeric M₁/M₁ muscarinic receptors have shown that modification of IL3 influences G protein selectivity, suggesting that IL3 has a critical role in receptor-G protein interaction (6–9). The importance of the C-terminal portion of IL3 in receptor conformation has been further indicated by the ability of mutations of specific residues in this region to cause constitutive activation of several adrenergic receptors (6–8).

Studies on spin-labeled double cysteine mutants of rhodopsin have indicated that rigid body motion of helix VI compared with helix VII is an important event in receptor activation (10) and that movement of the C-terminal region of IL3 occurs in constitutively active receptors (11). In the β₂-adrenergic receptor, substitution of the C-terminal region of IL3 with those of the α₁β₂ or α₂β₂-adrenergic receptors abolished the ability to couple to Gq (9, 12). In M₁, M₃, and M₅ muscarinic receptors, this region has been implicated in agonist-induced coupling to Gq,11 or Gi, respectively (13–15). Studies on chimeric human AT₁-and AT₂-Rs have demonstrated that the membrane proximal portions of IL3 are important for coupling to Gi (16). Also, the substitution of all charged residues in the C-terminal region of IL3 markedly reduces Ang II-induced inositol phosphate signal generation (17). Furthermore, exchange of this region of the AT₁-R with the corresponding sequence of the β₂-adrenergic receptor caused coupling of the receptor to Gi, as well as Gq during agonist stimulation (18). Other studies have demonstrated that a 16-amino acid peptide comprising the
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N-terminal portion of IL3 of the AT₁-R stimulates the binding of GTPγS to Gₐ (19).

Although these findings have clearly implicated the C-terminal region of IL3 in the coupling of the AT₁-R to Gₐ, the amino acids that are critical for this interaction have not been identified. To resolve this question, agonist-induced functional responses of mutant AT₁-Rs bearing replacements and deletions of amino acids in this region were determined in transiently transfected COS-7 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—DMEM, fetal bovine serum, and antibiotic solutions were from Biofluids (Rockville, MD). Ang II was from Sigma. [125I]-Ang II and [125I]-Sar¹, Ile⁸]Ang II were from Covance Laboratories (Vienna, VA) or NEN Life Science Products. myo-[2-3H]inositol was from Amersham Pharmacia Biotech. OptiMEM, inositol-free DMEM, and LipofectAMINE were from Life Technologies, Inc.

**Mutagenesis and Transient Expression of Mutant Rat AT₁-Rs—** Mutations were created using the Mutagene kit (Bio-Rad) in the sequence of the rat AT₁-R cDNA subcloned into the mammalian expression vector, pcDNA/Amp (Invitrogen, San Diego, CA), and mutated sequences were verified by dideoxy sequencing as described previously (20). Rat AT₁-R replacement mutant receptors are indicated by the single letter code of the original amino acid followed by the position number and the single letter code of the exchanged amino acid. Deletion mutant AT₁-R receptors are indicated by Del followed by the single letter code of the deleted amino acid and its position number. COS-7 cells were seeded at 5 × 10⁴ cells/well in 24-well culture plates in 1 ml of DMEM containing 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (COS-7 medium) and cultured for 3 days before transfection using 0.5 ml of OptiMEM containing 8 µg/ml of LipofectAMINE and the required DNA (usually 2 µg/ml) for 6 h at 37°C. Cells were cultured for 2 additional days in fresh COS-7 medium prior to use.

[Sar¹, Ile⁸]Ang II Binding to Intact Cells—To determine the expression level and structural integrity of the mutant receptor, the number of Ang II binding sites was determined by incubating the transfected cells with [125I]-Sar¹, Ile⁸]Ang II (0.05–0.1 µCi/sample) and increasing concentrations of unlabeled [Sar¹, Ile⁸]Ang II in DMEM for 6 h at 4°C. The bound radioactivity associated with the cells was measured by γ-spectrometry, and the binding curves were analyzed with the LIGAND computer program using a one-site model as described previously (21).

**AT₁-R Internalization Assay—**Internalization of mutant and wild-type AT₁-Rs were measured using 125I-Ang II at 37°C by γ-spectrometry, and values of the endocytotic rate constants (kₑ) were calculated as described previously (21). The percent of internalized ligand at each time point was calculated from the ratio of the acid-resistant specific binding to the total acid-resistant + acid-released (background) specific binding.

**Inositol Phosphate Measurements—**Transfected COS-7 cells in 24-well plates were labeled by incubation for 24 h in inositol-free DMEM containing 0.1% (w/v) bovine serum albumin, 2.5% (v/v) fetal bovine serum, antibiotics, and 20 µCi/ml myo-[2-3H]inositol. After washing and preincubation with 10 mM LiCl for 30 min, 1 µCi Ang II was added for a further 20 min, and incubations were stopped with perchloric acid. Inositol phosphates were extracted, the combined inositol bisphosphates + inositol trisphosphates fractions were separated using Bio-Rad AG 1-X8 columns, and their radioactivities were determined by liquid scintillation counting as described (22). At the expression levels used in this study, there was a linear relationship between cell surface receptor expression and the magnitude of agonist-stimulated inositol phosphate production (21).

**RESULTS**

**Binding Properties of Mutant AT₁-Rs—**To identify residue(s) in the C-terminal region of IL3 of the AT₁-R that are required for receptor activation, we analyzed a series of mutant receptors containing replacements or deletions of residues in the Lys²³²-Phe²³⁹ segment (Fig. 1). All mutants retained high binding affinity for [125I]-Sar¹, Ile⁸]Ang II (Table I), indicating their retention of the normal global conformation of the wild-type receptor. However, the mutant receptors displayed considerable variation in their expression levels compared with the wild-type receptor, and the expression of the Del N235 mutant receptor was markedly reduced (Table I).

**Inositol Phosphate Responses of Alanine Replacement and Deletion Mutant AT₁-Rs—**The ability of each mutant receptor to couple to Gₐ was evaluated by measuring the Ang II-induced stimulation of inositol phosphate production in transfected COS-7 cells. Alanine substitutions of the AT₁-R caused no major changes of the basal inositol phosphate levels (Fig. 2). The Ang II-stimulated inositol phosphate responses were significantly reduced in the D236A and D237A mutant receptor (Fig. 2, upper panel), but the expression levels of these mutant receptors were also lower (Table I). When the inositol phosphate responses were normalized to the number of expressed receptors, it was evident that the replacement of each residue in the Lys²³²-Phe²³⁹ segment by alanine had no significant effect on the ability of the receptor to couple to Gₐ and initiate signal transduction (Fig. 2, lower panel). Double alanine substitution of Lys²³² and Arg²³⁵ had no major effect on receptor expression (Table I) or inositol phosphate signal generation (Fig. 2). Triple alanine substitution of Asn²³³, Asp²³⁶, and Asp²³⁷ markedly decreased the expression level (Table I) and the InsP₃ responses of this mutant receptor (data not shown). The reduced inositol phosphate responses observed after triple alanine substitution of Ile²²⁸, Phe²³⁹ and Arg²⁴⁰ (Fig. 2, upper panel) could be attributed to the decreased expression level of this mutant receptor (Fig. 2, lower panel, Table I). These data indicate that negatively charged amino acids in this region are required for normal receptor expression and argue against the role of charged residues in inositol phosphate signal generation.

The C terminus of IL3 of GPCRs has been predicted to...
Fig. 2. Inositol phosphate responses of alanine replacement mutant AT$_{1a}$-Rs. Upper panel, [³H]inositol-labeled COS-7 cells expressing the wild-type (WT), the indicated point mutant, the K232A/R234A (KR/AA) double alanine substitution mutant, or the I238A/F239A/R240A (IFR/AAA) triple alanine substitution mutant AT$_{1a}$ receptors were preincubated with 10 mM LiCl for 30 min prior to the addition of Ang II. After incubation for 20 min in the absence (open bars) or presence of 1 μM Ang II (hatched bars) the radioactivity of [³H]inositol phosphates was measured as described under “Experimental Procedures.” Lower panel, inositol phosphate responses were normalized to the number of expressed receptors (Table I) and are shown as a percentage of the wild-type response. The data represent mean ± S.E. from three independent experiments each performed in duplicate. 

Table I

| AT$_{1a}$R         | $K_d$ (nM) (%) of wild-type | $B_{max}$ (pmol/mg of protein) |
|-------------------|-----------------------------|-------------------------------|
| Wild-type         | 1.75 ± 0.24                 | 100.0                         |
| K232A             | 2.35 ± 0.50                 | 117.4 ± 24.1                  |
| P233A             | 1.88 ± 0.50                 | 91.1 ± 5.2                    |
| R234A             | 1.71 ± 0.39                 | 53.1 ± 1.0                    |
| N235A             | 1.74 ± 0.18                 | 44.7 ± 2.4                    |
| D236A             | 2.13 ± 0.65                 | 23.5 ± 1.5                    |
| D237A             | 2.96 ± 0.48                 | 17.1 ± 0.1                    |
| K232A/R234A       | 1.52 ± 0.41                 | 110.4 ± 5.9                   |
| N235A/D236A/D237A | 1.38 ± 0.35                 | 5.3 ± 1.9                     |
| I238A/F239A/R240A | 1.47 ± 0.44                 | 12.1 ± 0.3                    |
| I238A             | 1.45 ± 0.11                 | 128.0 ± 5.7                   |
| I238S             | 1.55 ± 0.15                 | 146.3 ± 15.4                  |
| I238K             | 1.60 ± 0.18                 | 99.6 ± 5.2                    |
| I238D             | 1.80 ± 0.22                 | 240.0 ± 4.4                   |
| F239A             | 1.20 ± 0.06                 | 25.6 ± 2.7                    |
| F239L             | 1.66 ± 0.48                 | 138.2 ± 36.6                  |
| F239N             | 1.12 ± 0.22                 | 126.7 ± 45.1                  |
| F239S             | 1.94 ± 0.15                 | 87.8 ± 1.8                    |
| F239Y             | 1.21 ± 0.17                 | 75.3 ± 24.1                   |
| F239K             | 0.96 ± 0.24                 | 31.3 ± 0.3                    |
| F239R             | 2.55 ± 0.07                 | 36.3 ± 0.8                    |
| F239D             | 1.56 ± 0.04                 | 91.6 ± 0.3                    |
| Del P233          | 1.69 ± 0.56                 | 103.3 ± 10.3                  |
| Del R234          | 2.05 ± 0.58                 | 98.5 ± 2.1                    |
| Del N235          | 1.53 ± 0.06                 | 0.4 ± 0.2                     |
| Del D237          | 1.28 ± 0.26                 | 44.6 ± 3.2                    |
| Del D238          | 1.84 ± 0.39                 | 139.8 ± 6.2                   |
| Del F239          | 1.88 ± 0.14                 | 48.0 ± 3.6                    |
| Del D240          | 4.43 ± 1.06                 | 30.0 ± 0.3                    |

Inositol Phosphate Responses of Ile$^{238}$ and Phe$^{239}$ Substitution Mutant AT$_{1a}$-Rs—In adrenergic receptors, substitutions of amino acids in the location corresponding to position 239 of the AT$_{1a}$-R cause constitutive activation of the mutant receptors (24). A more detailed analysis of the structural requirements in positions 238 and 239 was performed to study the role of this region in AT$_{1a}$-R activation. Replacement of Phe$^{239}$ with other apolar amino acids such as alanine and leucine or with tyrosine, a polar residue, had little or no effect on inositol phosphate signal generation (Fig. 4). However replacement with asparagine or serine significantly impaired the signaling ability of the AT$_{1a}$-R. Replacement of Phe$^{239}$ with the acidic residue, aspartate, had the most profound effect and reduced inositol phosphate production by about 90%. In contrast, replacement of Phe$^{239}$ with basic residues (lysine or arginine) significantly enhanced inositol phosphate responses. None of the tested mutants had a major effect on inositol phosphate levels in unstimulated cells, indicating that, in contrast to the adrenergic receptor(s), replacement of the amino acid in this position with other residues does not cause constitutive activation of the AT$_{1a}$-R (Fig. 4, Table I).

Replacement of Ile$^{238}$ with polar or charged amino acids also affected the signal generation efficacy of the AT$_{1a}$-R (Fig. 5). Substitution of Ile$^{238}$ with apolar alanine had no effect on inositol phosphate responses, but replacement with polar serine impaired inositol phosphate production when the data were normalized to the expression levels of the receptor (Fig. 5, lower panel). Replacement of Ile$^{238}$ with aspartate considerably increased the expression of the receptor in COS-7 cells (Table I). However, despite the increased expression level of the 1238D receptor its inositol phosphate responses were significantly impaired and when normalized for expression were reduced by ~85%. Substitution of Ile$^{238}$ with lysine, a basic residue, had no effect on the expression level of the receptor, but the inositol phosphate responses of the 1238K receptor were significantly reduced (Fig. 5, Table I).

assume an α-helical structure that is continuous with the sixth transmembrane helix (23). Because deletions of individual residues within a helix change the orientation of the remaining amino acids, such mutations are likely to modify or abolish functionally important contacts by the constituent residues of the helix. When single amino acids in the C-terminal region of IL3 were deleted to identify functionally important residues, only the removal of Asn235 reduced the expression of the AT$_{1a}$-R to such an extent that normalization of its inositol phosphate response was not reliable. Deletion of residues Arg$^{240}$, Phe$^{239}$, and Ile$^{238}$, adjacent to the sixth intramembrane helix, markedly impaired Ang II-induced inositol phosphate generation (Fig. 3). However, deletion of Asp$^{237}$, Arg$^{234}$, or Pro$^{233}$, residues that are more distant from helix 6, had no effect on the inositol phosphate responses (Fig. 3). These findings suggest that the residue located in position 238 is an important structural determinant of Ang II-induced signal generation.

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Replacement of Ile$^{238}$ with polar or charged amino acids also affected the signal generation efficacy of the AT$_{1a}$-R (Fig. 5). Substitution of Ile$^{238}$ with apolar alanine had no effect on inositol phosphate responses, but replacement with polar serine impaired inositol phosphate production when the data were normalized to the expression levels of the receptor (Fig. 5, lower panel). Replacement of Ile$^{238}$ with aspartate considerably increased the expression of the receptor in COS-7 cells (Table I). However, despite the increased expression level of the 1238D receptor its inositol phosphate responses were significantly impaired and when normalized for expression were reduced by ~85%. Substitution of Ile$^{238}$ with lysine, a basic residue, had no effect on the expression level of the receptor, but the inositol phosphate responses of the 1238K receptor were significantly reduced (Fig. 5, Table I).
Internalization of Mutant AT$_{1a}$-Rs—Individual replacements of each amino acid in the Lys$_{232}$-Ile$_{238}$ segment with alanine had no major effect on the agonist-induced internalization kinetics of the AT$_{1a}$-R in COS-7 cells, but replacement of Phe$_{239}$ with alanine modestly reduced the internalization rate of the receptor (Fig. 6). Other substitutions of Ile$_{238}$ and Phe$_{239}$ caused impairment of the internalization kinetics of the mutant receptors (Fig. 7) that paralleled their reductions in inositol phosphate generation (Fig. 2). Replacement of Ile$_{238}$ with alanine had no effect on the internalization kinetics, but substitution with charged residues caused a significant decrease in the internalization kinetics of the receptor (Fig. 7 and Table II). Compared with the wild-type AT$_{1a}$-R, the $K_e$ values of the I238K and I238D mutant receptors were reduced to 45 ± 2% and 10 ± 0.1% of the wild-type value, respectively. The $K_e$ values of mutant receptors in which Phe$_{239}$ was replaced by other residues (F239K, F239R, F239Y, F239A, and F239L) were 132 ± 1.8%, 79.4 ± 0.7%, 85.8 ± 6.4%, 60.6 ± 8.3%, and 66.3 ± 8.6% of that of the wild-type receptor, respectively. In contrast, the $K_e$ values of the F239S, F239N, and F239D mutant receptors were reduced to 4.5 ± 2.1%, 6.1 ± 3.5%, and 2.9 ± 1.0% of that of the wild-type receptor, respectively (Fig. 7 and Table II).

DISCUSSION

The N- and C-terminal portions of IL3 have been implicated in G protein coupling to several seven transmembrane domain receptors (25–27). Discontinuous sequences of IL3, IL2, and the C-terminal cytoplasmic tail have been predicted to comprise an agonist-induced conformation-dependent surface that permits interaction with G proteins (28). Earlier studies using chimeric AT$_{1a}$-Rs (16) and substitutions of charged residues in the C-terminal portion of IL3 (17) suggested that this region of the receptor is also required for Ang II-induced signal generation. In addition to activating G protein-dependent second messenger systems, the AT$_{1a}$-R also undergoes phosphorylation and rapid internalization after agonist stimulation (29–32). Substitution of amino acids 234–240 of the AT$_{1a}$-R with the corresponding sequence of the $b_2$ receptor impaired its internalization kinetics, suggesting that this region has a role in the agonist-induced internalization of the receptor (18). However, alanine substitutions of individual amino acids in the C-terminal region of IL3 did not affect the internalization kinetics of the AT$_{1a}$-R, with the exception of the F239A mutation, which reduced the internalization kinetics in a moderate extent. Studies on the effects of replacing the distal segment of IL3 of the AT$_{1a}$-R with the corresponding regions of the $\alpha_1$-adrenergic, $b_2$-adrenergic, and AT$_{2}$ receptors revealed that the RNDDIFR sequence (from Arg$_{234}$ to Arg$_{240}$) of the AT$_{1a}$-R has a major role in G protein coupling and receptor signaling (18). In the present work, alanine substitutions or deletions of Arg$_{234}$, Aan$_{235}$, Asp$_{236}$, or Asp$_{237}$ had no significant effect on the inositol phosphate signaling or internalization kinetics of the receptor, but individual deletions of Ile$_{238}$, Phe$_{239}$, or Arg$_{240}$...
markedly attenuated inositol phosphate production. Because the C-terminal region of IL3 is believed to form an extension of the α-helical structure of the sixth transmembrane helix, this effect of amino acid deletions could result from changes in the position of Ile238 relative to the rest of the helix. The lack of effect of alanine substitutions raises the possibility that, similar to the role of Leu222 in the N-terminal region of IL3 (33, 34), apolar amino acids in this region are favorable for signal generation.

Although substitutions with alanine or other apolar amino acids were tolerated, replacements of Ile 238 with polar or charged amino acids significantly impaired the Ang II-induced inositol phosphate responses of the AT1a-R. These data suggest that the presence of a hydrophobic amino acid at position 238 is required for AT1-R activation. It has been reported that the four amino acid sequence, VTIL (Val385, Thr386, Ile389, and Leu390) located at the boundary of IL3 and helix 6, determines the Gαi coupling specificity of the M2 muscarinic receptor. Also, this region of the M2 receptor has been proposed to interact with the C-terminal domain of Gαi5 (15, 35). The corresponding AALS motif ( Ala488, Ala489, Leu492, and Ser493) of the Gq-coupled M3 muscarinic receptor together with IL2 and the N-terminal part of IL3 were suggested to be directly involved in coupling to Gq (36). The position of Ile238 of the AT1-R corresponds to the position of Val385 of the M2 receptor and Ala488 of the M3 receptor. As in the muscarinic receptors, this region appears to be an important determinant of G protein activation of the AT1-R.

In addition to their effects on inositol phosphate signal generation, mutations of Ile238 also impaired the internalization kinetics of the AT1-R. Although the agonist-induced internalization of GPCRs often occurs in parallel with G protein activation, mutational analysis of the AT1 and other receptors has shown that the structural requirements of these processes are distinct, and AT1-R internalization can occur in the absence of inositol phosphate signal generation and G protein coupling (20, 31, 32, 37, 38). Because receptor internalization does not depend on G protein-mediated signaling, the inhibitory effects of substituting Ile238 with polar and charged amino acids on receptor internalization are independent of G protein coupling. Although it cannot be excluded that Ile238 directly couples the receptor to G protein activation and internalization, these data suggest that Ile238 is required for a step in receptor activation that is proximal to receptor G protein coupling. The most likely mechanism is the participation of Ile238 in an intramolecular interaction that is required for the active conformation of the receptor. Such a mechanism is consistent with a recent report.
on the mapping of G protein binding domains of the AT1a-R (39). In contrast to peptides derived from the N-terminal region of IL2 and IL3 and the juxtamembranous region of the cytoplasmic tail, peptides derived from the C-terminal region of IL3 did not compete for receptor G protein coupling (39). The proposed participation of Ile238 in an intramolecular interaction is also in agreement with the GPCR model of Baldwin (40), which predicts that the side chain of the amino acid in the position of Ile238 faces helix 7 and thus points away from the putative G protein binding region (28, 40).

In view of the requirement for an apolar amino acid in the position of Ile238 in the AT1a-R, the properties of the amino acids in this location in other members of GPCR family I were examined. Among 171 GPCR sequences from the data base of comprehensive alignment of G protein-coupled receptor sequences compiled by the Molecular Recognition Section of the National Institutes of Health, only 22 receptors contain polar amino acids at this position. These residues were 15 threonines, 4 serines, 1 asparagine, and 2 histidines. All other members of the GPCR family I contain an apolar residue at this position. The frequent occurrence of a corresponding apolar amino acid in GPCRs raises the possibility of a general requirement for such residues in GPCR activation by agonist ligands.

The position of Phe239 in the AT1-R has a different amino acid requirement compared with that of position 238. The location of this amino acid corresponds to Thr386 in the VTIL motif of the M2 muscarinic receptor and Ala489 in the AALS motif of the M4, M5, and M6 muscarinic receptors. In addition, Phe239 corresponds to a position that frequently causes constitutive activation if substituted in adrenergic receptors (24). However, a completely different mechanism of constitutive activation has been reported for the AT1-R (41). In contrast to Ile238, replacement of Phe239 with polar residues had a variable effect on AT1-R activation. Substitutions of apolar amino acids or tyrosine caused only minor changes in signal generation and internalization of the receptor, but substitution of asparagine or serine decreased these responses. Although substitution of a negatively charged aspartic acid for Phe239 severely impaired inositol phosphate generation and receptor internalization, replacement by positively charged residues increased these responses. Recent studies have predicted that AT1-R activation involves an outward movement of the cytoplasmic end of the sixth transmembrane helix, accompanied by a clockwise rotation (as viewed from the cell interior) by ~30° (42). A possible explanation for the opposite effects of basic and acidic amino acids is that positively charged residues facilitate this movement, whereas the presence of a negative charge interferes with it. Similar to the results obtained with mutations of Ile238, substitution of Phe239 caused a parallel impairment of inositol phosphate signal generation and receptor internalization. These data suggest the Phe239, like Ile238, has a role in the formation of the active conformation of the receptor. This conclusion is in accordance with the results of studies on rhodopsin-transducin interaction that showed that substitution of the amino acids (Val352 and Thr351) of rhodopsin that correspond to Ile238 and Phe239 of the AT1a-R interfere with the formation of the active (M II) state of the photoreceptor and thus indirectly affect its interaction with transducin (43).

In summary, this study demonstrates the importance of two specific residues, Ile238 and Phe239, in the C terminus of IL3 in the mechanism of activation of the AT1-R. The parallel impairment of receptor signaling and internalization suggests that these adjacent apolar residues may participate in intramolecular interaction(s) that are required for receptor activation. The exact mechanism of this interaction and its role in other GPCRs requires further investigation. However, based on the present findings, and other evidence that implicates this region of GPCRs in signal transduction, it is possible that these amino acids and their counterparts have a general role in receptor activation by agonist ligands.

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