Mutation of Asn<sup>111</sup> in the Third Transmembrane Domain of the AT<sub>1A</sub> Angiotensin II Receptor Induces Its Constitutive Activation<sup>g</sup>

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A preliminary model of the rat AT<sub>1A</sub> angiotensin II (AII) receptor (Joseph, M. P., Maigret, B., Bonnafous J.-C., Marie, J., and Scheraga, H. A. (1995) J. Protein Chem. 14, 381–398) has predicted an interaction between Asn<sup>111</sup> located in transmembrane domain (TM) III and Tyr<sup>292</sup> (TM VII) in the nonactivated receptor; a disruption of this interaction upon AII activation would allow Tyr<sup>292</sup> to interact with the conserved Asp<sup>74</sup> (TM II). The previous verification that Tyr<sup>292</sup> is essential for receptor coupling to phospholipase C (Marie, J., Maigret, B., Joseph, M. P., Larguier, R., Nouet, S., Lombard, C., and Bonnafous, J.-C. (1994) J. Biol. Chem. 269, 20815–20818) prompted us to check the possible alterations in receptor properties upon Asn<sup>111</sup> → Ala mutation. The mutated receptor (N111A) displayed: (i) strong constitutive activity, with amplification of the maximal phospholipase C response to AII; (ii) agonist behavior of the AT<sub>2</sub>-specific ligand CGP 42112A, [Sar<sup>1</sup>,Ile<sup>8</sup>]AII, and [Sar<sup>1</sup>,Ala<sup>8</sup>]AII, antagonists of the wild-type receptor; (iii) inverse agonism behavior of the non-peptide ligands DuP 753, LF 7-0156, and LF 8-0129. The results are discussed in the light of the allosteric ternary complex models and other described examples of constitutive activation of G protein-coupled receptors.

Constitutive activation of G protein-coupled receptors, initially reported by Cotechia et al. (1) for the α<sub>1</sub>-adrenergic receptor, is now well documented and has been extended to many other members of this large family (2–21). Moreover, mutations inducing receptor constitutive activation have been found to be associated with human diseases (13–16). Data on the β<sub>2</sub>-adrenergic receptors have prompted their authors to propose an extended version of the ternary complex model, based on the existence of active and inactive receptor states (4, 22). The same mechanistic model, either in its initial form (22) or in a refined version (23), have provided interpretations for the “negative antagonism” or “inverse agonism” phenomenon, evidenced for ligands of β<sub>2</sub>-adrenergic (24, 25), B<sub>2</sub> bradykinin (26), m5 muscarinic (21), and thyrotrphin-releasing hormone (18) receptors. As emphasized by the recent work of Cotechia’s group on the α<sub>1H</sub>-adrenergic receptor (8), a correlation between mechanistic considerations and molecular events associated to receptor conformational changes is obviously required. The work reported in the present paper is based on a previous molecular modeling study (27) which aimed at predicting modifications of specific amino acid side chain interactions during the process of the angiotensin II type 1 (AT<sub>1</sub>)<sup>1</sup> receptor activation. This preliminary model (27) postulated that an interaction between Asn<sup>111</sup> (TM III) and Tyr<sup>292</sup> (TM VII) might exist in the non-activated receptor and that activation by AII would induce its disruption to allow Tyr<sup>292</sup> to interact with the conserved Asp<sup>74</sup> (TM II). Previous experimental work from this laboratory is consistent with an essential role of a Tyr<sup>292</sup>-Asp<sup>74</sup> interaction in the transduction mechanism (28). The model prompted us to check whether the absence of Asn<sup>111</sup> would favor this interaction, leading to constitutive receptor activity. In this paper we demonstrate that the N111A mutant receptor displays a strong constitutive activity as well as striking pharmacological changes. The results are discussed in the light of above-mentioned current models (22, 23).

**MATERIALS AND METHODS**

**Reagents**

AII, [Sar<sup>1</sup>l]AII, [Sar<sup>1</sup>l,Ile<sup>8</sup>]AII, and [Sar<sup>1</sup>l,Ala<sup>8</sup>]AII were purchased from Bachem (Budendorf, Switzerland). CGP 42112A was provided by Drs. M. De Gasparo and S. Bottari (Ciba Geigy, Basel, Switzerland). Non-peptide antagonists LF 7-0156, LF 8-0129, and DUP 753 were synthesized by Fournier Laboratoires (Daix, France). AII, [Sar<sup>1</sup>l]AII, and CGP 42112A were radiolabeled as described previously in Refs. 29 and 30, respectively. myo-[3H]-Inositol was from DuPont NEN. COS-7 cells were from the European Cell Type Collection.

**Site-directed Mutagenesis and Expression**

The amino acid mutation Asn<sup>111</sup> → Ala was carried out as described previously (28). The cDNA sequences of the wild type and N111A mutant rat AT<sub>1A</sub> receptors were subcloned in the XbaI site of the polylinker of the eukaryotic expression vector pCMV (31). Receptors were transiently expressed in COS-7 cells by using the electroporation transfection method: 10<sup>6</sup> cells were resuspended in 300 μl of electroporation buffer (50 mM K<sub>2</sub>HPO<sub>4</sub>, 20 mM CH<sub>3</sub>COOK, 20 mM KOH, pH 7.40) and incubated for 10 min at room temperature in an electroporation cuvette (0.4 cm electrode gap, Bio-Rad) with 20 μg of pCMV carrier and different amounts of pCMV containing cDNA receptors sequences (30–300 ng range). They were submitted to an electric discharge (950 microfarads, 280 V, 50 μs), then cultured for 2 days at 37 °C in Dulbecco’s modified Eagle’s medium, 4.5 g/liter glucose, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin before binding or IP accumulation experiments.

<sup>1</sup>The abbreviations used are: AT<sub>1</sub>, receptor type 1 angiotensin II receptor; AT<sub>2</sub>, receptor type 2 angiotensin II receptor; AII, angiotensin II; Sar, sarcosine; GTP-γS, guanosine 5′-O-(3-thiotriphosphate); IP<sub>3</sub>, inositol phosphate; PBS, phosphate-buffered saline; WT, wild type; TM, transmembrane domain; GPCR, G protein-coupled receptor.
Binding Assays

**Plasma Membrane—Crude membranes from COS-7 cells transiently expressing the wild type or mutant AT1A receptors were prepared according to Ref. 28.** Protein concentrations were measured as described in Ref. 32. 125I-AII or 125I-CGP 42112A binding assays were performed as described previously (33). In some experiments involving GTPγS treatment, the membranes were washed three times with binding buffer (50 mM Na2HPO4, 5 mM MgCl2, 1 mg/ml bovine serum albumin, 1 mg/ml bacitracin, pH 7.40) supplemented with 200 mM NaCl, incubated for 10 min at 30°C in the presence or absence of 100 µM GTPγS before binding experiments carried out in the same medium.

**Intact Cells—** [Sar1]AII binding to transfected COS-7 cells grown in 6-well tissue culture clusters (5 × 10⁵ cells/well) was effected as in Ref. 28.

**Inositol Phosphate Assays**

COS-7 cells expressing the wild type or N111A mutant receptors were grown in 6-well tissue culture clusters and labeled for 24 h with [2-3H]inositol (1.5 mM/ml, 1 µCi/m) in minimum essential medium deprived of serum and unlabeled inositol. Before stimulation, cells were incubated at 37°C for 1 h in Dulbecco’s phosphate-buffered saline (pH 7.40). After a 15-min LiCl treatment, cells were incubated in the presence or absence of peptide ligands for 15 min at 37°C in Dulbecco’s PBS supplemented with 10 mM LiCl, 1 mg/ml bovine serum albumin, and 1 mg/ml bacitracin. LiCl was omitted in short-time [Sar1]AII stimulation experiments (15 and 30 s). For experiments involving non-peptide ligands, cells were incubated, after the 1-h PBS incubation, in the presence or absence of non-peptide compounds for 30 min at 37°C in Dulbecco’s PBS supplemented with 10 mM LiCl and 0.1 mg/ml bacitracin without LiCl pretreatment. Pooled inositol phosphates (IPs) were extracted and measured as described previously (28, 34).

**RESULTS**

**Constitutive Activation of the N111A Mutant AT1A Receptor** —The detailed analysis of basal inositol phosphate production, in the presence of Li⁺ ions, by the WT or N111A receptors transiently expressed in COS-7 cells revealed strong constitutive activation of the N111A mutant AT1A receptor (Fig. 1A). A significant difference between basal IP production by the WT and the N111A mutant receptors was detected for expression levels of about 10⁵ sites/cell. This constitutive activation might be overlooked in the cases of lower expression levels. In this respect, the electrophoretic technique is well adapted to the provision of homogeneous cell transfection over a wide range of expression levels. The maximal extent of [Sar1]AII stimulation of IP production was greatly potentiated in the N111A mutant receptor (Fig. 1B). This potentiation was evident at stimulation times as short as 15 s (Fig. 1C), which indicates that it resulted from an intrinsic activation property of the receptor rather than desensitization or sequestration phenomena. In addition, these short-time experiments carried out in the absence of Li⁺ ions showed that the steady-state levels of IPs are similar in the WT and mutant receptor (Fig. 1C, t = 0), the constitutive activation requiring the addition of IP₃, phosphatase inhibitors. The K_m values for the N111A mutant receptor was not significantly altered: 2.88 ± 1.03 nM versus 1.03 ± 0.40 nM for the WT receptor (Fig. 2) which is consistent with K_m values for [125I-Sar1]AII binding (1.1 ± 0.2 nM and 0.76 ± 0.10 nM for N111A and WT receptors, respectively) (35). In order to dissect the changes in receptor coupling properties upon Asn111 → Ala replacement, we checked the effect of GTPγS, a non-hydrolyzable analog of GTP, on the affinities of the WT and mutant receptor for [125I-Sar1]AII. The expected decreased in WT receptor affinity (increase in K_m value from 1.34 ± 0.23 nM to 4.31 ± 0.65 nM) was no longer observed for the constitutively active mutant receptor (K_m values: 2.12 ± 0.36 nM and 1.84 ± 0.11 nM in the absence and presence of GTPγS, respectively) (Fig. 3). These data concerning the comparison of binding affinities and K_m values for the WT and N111A receptors (which contrast with other examples of constitutive activation of G protein-coupled receptors (1, 3–5, 7, 18, 21)) and the effects of GTPγS treatment on agonist binding will be discussed later in the light of published mechanistic models (22, 23).

**Changes in Pharmacological Properties of the AT1A Receptor upon Asn¹¹¹ → Ala Mutation** —We noted earlier (35) that the N111A mutant receptor displayed an increased affinity for the AT1-specific ligand CGP 42112A. Direct binding of 125I-CGP 42112A to membranes from COS-7 cells expressing the N111A mutant receptor led to a K_i value of 96 nM, in agreement with a previously determined K_i value (35). Moreover, CGP 42112A displayed a marked partial agonist activity for the N111A mutant receptor (maximal response: 27% of the response to [Sar1]AII (Fig. 4A) with a K_act value of 42 nM (Fig. 4C). The other peptidic compounds [Sar⁺,Ile⁶]AII and [Sar⁺,Ala⁸]AII, which possess unchanged affinities for the mutant receptor (35) and are devoid of agonist activity for the recombinant WT receptor, also behaved as partial agonists for the N111A mutant (Fig. 4A) (maximal activities: 52% and 27%, respectively,
of the response induced by [Sar^1]AII. We previously demonstrated that Asn^{111} to Ala replacement induced a strong decrease in receptor affinity for all tested non-peptide ligands: DUP 753, LF 7-0156, and LF 8-0129 (35). In spite of these reduced affinities, we could check the ability of these ligands to modulate the IP responses of the WT and N111A mutant receptors. While the activity of the WT receptor was not significantly affected, a marked decrease in IP production was observed upon treatment with the three non-peptide ligands (Fig. 4B). These inverse agonism properties were characterized by $K_{act}$ values of 2.4 $\mu$M, 0.4 $\mu$M, and 0.38 $\mu$M for DUP 753, LF 7-0156, and LF 8-0129, respectively (Fig. 4C).

**DISCUSSION AND CONCLUSION**

The data reported in this paper demonstrate striking changes in the properties of the rat AT_{1A} receptor induced by Ala replacement of the Asn^{111} residue, located in the third transmembrane domain: constitutive activation of basal IP production and amplification of the response to angiotensin II; induction of inverse agonism properties of the non-peptide ligands DUP 753, LF 7-0156, and LF 8-0129, evidenced in spite of their greatly decreased affinities for the mutated receptor (35); induction of partial agonist properties of the AT_{2}-specific peptide ligand CGP 42112A, associated with an increased affinity, as well as [Sar^1,Ile^8]AII and [Sar^1,Ala^8]AII derivatives which are antagonists of the wild-type receptor.

We tried to correlate these observations with mechanistic interpretations of the "allosteric ternary complex" models previously proposed by Lefkowitz's group (22) and discussed and refined by Kenakin (23) (Fig. 5). Classical simulation curves...
Acid(s) might act as a relay in the cascade of conformational time to exclude the possibility that one (or several) other amino acid(s) might act as (a) relay(s) in the cascade of conformational changes leading to receptor coupling to a specific G protein.

Many examples of constitutive activity of G protein-coupled receptors have been reported. It appeared possible to induce constitutive activation of numerous members of this family by mutation of amino acids located at various receptor positions (1–21). The problem of molecular event conservation in activation processes of GPCR is raised. It can be envisaged that some of these events are conserved, while others are specific to some receptors or some subfamilies. A recent work on the α-1B receptor has emphasized the role of conserved polar amino acids in receptor activation, the mutation of some of these residues leading to constitutively active receptors. The dissection of conformational changes underlying receptor activation and the prediction of the molecular nature of receptor-activated states have been attempted through the use of molecular dynamics (8). The Asn111 residue of the AT1A receptor, the mutation of which induces constitutive activation, is found at homologous positions in other peptide hormone receptors: AT2 and Xenopus angiotensin receptors, bradykinin, opioid, interleukin 8, and somatostatin receptors for instance. It is noticeable that mutation of Cys126 (7) in the α-1B adrenergic receptor, which occupies a position homologous to that of Asn111 in the AT1A receptor, also induces constitutive activation. Tyr292 of the AT1A receptor, which is postulated to interact with Asn111 in the non-activated receptor, is conserved in all GPCR. More direct proof for Asn111-Tyr292 interaction are obviously required, even if the lack of tyrosine conservation is not necessarily inconsistent with the model. Nevertheless, in the absence of high resolution crystallographic data for GPCR, mutagenesis experiments which lead to constitutive activation can support hypotheses about receptor stabilization through interaction between specific residues and their rearrangements upon hormonal activation. Data about rhodopsin (10, 11) suggest that disruption of a critical salt bridge between Lys296 (TM VII) and Glu113 (TM III) induces constitutive activation. They provide insight into structural features inasmuch as they can provide information on the relative spatial positions of the transmembrane α helices and interactions between them.

FIG. 6. Molecular modeling of WT and N111A AT1A receptors. The model of N111A mutant receptor has been built by Asn111 → Ala replacement in the previously described model of WT receptor (27). The model of WT receptor (left panel) predicts a possible hydrogen bond between Asn111 and Tyr292 in the non-activated receptor, the hormone activation resulting in disruption of this interaction to allow Tyr292 to interact with Asp74. Suppression of Asn111 (right panel) would favor this latter interaction in the absence of hormone, thus inducing constitutive activation. A molecular dynamic study has been shown to be consistent with this hypothesis (Silicon Graphics Indigo2 workstation, DISCOVER 95.0 software; non-bonded cut-off distance: 12 Å, dielectric constant ε = 1; C-α fixed; dynamic run: 80 ps, 310 K).
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