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Functional interactions of L-162,313 with angiotensin II receptor subtypes and mutants

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Abstract

A nonpeptide ligand, L-162,313 (5,7-dimethyl-2-ethyl-3-[[4-[2(*n*-butyloxycarbonylsulfonamido)-5-isobutyl-3-thienyl]phenyl]methyl]imidazo[4,5,6]pyridine) was characterized on the angiotensin II receptors. This compound displaced [^{125}I][Sar1]angiotensin II from rat angiotensin AT_{1A}, AT_{1B} or AT_2 receptor individually expressed in COS-7 cells (K_i = 207 nM, 226 nM and 276 nM, respectively). In monkey kidney cells expressing angiotensin AT_{1A} or AT_{1B} receptors, it stimulated inositol phosphate accumulation, but the maximal response was 34.9 and 23.3%, respectively, of those of angiotensin II. Furthermore, an antagonist effect of L-162,313 was observed in response to angiotensin II. Single-point substitutions in the second and third transmembrane domains of the rat angiotensin AT_{1A} receptor, which impaired the binding of losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1[(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole), also affected the binding of L-162,313. Losartan and L-162,313 require some common structural determinants for non-peptide recognition on the angiotensin AT_1 receptor. Furthermore, some of these substitutions, which impaired the inositol phosphate accumulation in response to angiotensin II, also impaired the response to L-162,313. Angiotensin II and L-162,313 require common critical residues for angiotensin AT_1 receptor activation. © 1998 Elsevier Science B.V.

Keywords: Angiotensin II; Losartan; Angiotensin AT_1 receptor agonist; L-162,313; Nonpeptide ligand; Site-directed mutagenesis; Binding site; G protein coupled receptor; Signaling

1. Introduction

Two pharmacologically distinct angiotensin II receptor types have been defined and designated angiotensin AT_1 and AT_2 receptors (Chiu et al., 1989; Whitebread et al., 1989). Angiotensin AT_1 receptors bind biphenylimidazole antagonists such as losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1[(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) with high affinity, whereas angiotensin AT_2 receptors bind pentapeptide analogue CGP42112A (nicotinic acid-Tyr-(N^{Σ}-benzyloxycarbonyl-Arg)Lys-His-Pro-Ile-OH) and nonpeptide ligands such as PD123177 (1-(4-amino-3-methylphenyl)methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5c] pyridine-6-carboxylic acid 2HCl) with high affinity. Cloning of these receptor complementary DNAs, in the rat particularly, has revealed that they both belong to the seven transmembrane domain receptor family (Murphy et al., 1991; Kambayashi et al., 1993; Mukoyama et al., 1993). Two closely related angiotensin AT_1 isoforms (AT_{1A} and AT_{1B}) have been identified in the rodent species (Iwai and Inagami, 1992; Sandberg et al., 1992).

Agonist binding to the angiotensin AT_1 receptor induces a conformational change of the receptor leading to receptor activation and subsequent coupling to heterotrimeric G proteins. The main pathway involved in angiotensin AT_1 receptor signaling requires the coupling to a Gq family member, which activates β-phospholipase C. This activation leads to the hydrolysis of a membrane phospholipid producing two second messengers, inositol-1,4,5 trisphosphate, which mobilizes calcium from intracellular stores and 1,2-diacylglycerol, which activates members of the protein kinase C family (Catt et al., 1988). A large majority, if not all, of the physiological actions of angiotensin II are mediated by the angiotensin AT_1 receptors (Timmermans et al., 1993), whereas the AT_2 intracellular signaling pathways, like their physiological actions, are still a matter of debate.

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Nonpeptide ligands for peptide receptors belonging to the seven transmembrane domain receptor family are usually antagonists. However, it was recently observed that L-162,313 (5,7-dimethyl-2-ethyl-3-[[4-[2(*n*-butyloxycarbonylsulfonamido)-5-isobutyl-3-thienyl]phenyl] methyl]imidazo[4,5,6]-pyridine), a nonpeptide compound mimics the biological activity of angiotensin II in vivo, by increasing the duration of the pressor response in comparison to that observed with angiotensin II in the rat (Kivlighn et al., 1995).

In order to determine the pharmacological and functional properties of this compound, we examine, in this study, how this biphenylimidazole derivative interacts with rat angiotensin II types (AT_1 and AT_2) and subtypes (AT_{1A} and AT_{1B}) in transiently transfected monkey kidney cells, and how this compound stimulates inositol phosphate production in comparison to the activation observed with angiotensin II. Furthermore, some polar residues present in the second and third transmembrane domains of the rat angiotensin AT_{1A} receptor are required for the binding of non- or pseudo-peptide compounds (i.e., losartan and CGP42112A, respectively) and functional coupling to a Gq family member (Bihoreau et al., 1993; Monnot et al., 1996). Therefore, seven single-point mutants of the angiotensin AT_{1A} receptor ([$D^{74}N$].AT_{1A}, [$D^{74}E$].AT_{1A}, [$S^{105}A$].AT_{1A}, [$S^{107}A$].AT_{1A}, [$S^{109}A$].AT_{1A}, [$N^{111}A$].AT_{1A} and [$S^{115}A$].AT_{1A}) are studied in this report, in order to determine the similarities between the losartan and the L-162,313 binding sites and the involvement of some residues in its agonist (or antagonist) activity.

2. Materials and methods

2.1. Materials

L-162,313 was a gift from Merck Sharp and Dohme (Rahway, NJ, USA). [Sar1]angiotensin II and angiotensin II were purchased from Sigma (St. Louis, MO, USA). The nonpeptide compound was dissolved in 100% dimethyl sulfoxide (10^{-2} M stock solution) and further diluted in the assay buffer. *Myo*-[^{3}H]inositol was purchased from Amersham (Bucks, UK).

2.2. Methods

2.2.1. Cell culture and transfections

The wild-type or mutated angiotensin II receptor cDNAs subcloned in the expression vector pECE (Ellis et al., 1986) were obtained as previously described: for the rat wild-type angiotensin AT_{1A} receptor cDNA and the two mutants [$D^{74}N$].AT_{1A} and [$D^{74}E$].AT_{1A} (Bihoreau et al., 1993); for the rat wild-type AT_{1B} receptor cDNA (Conchon et al., 1994); for the five rat angiotensin AT_{1A} mutants [$S^{105}A$].angiotensin AT_{1A}, [$S^{107}A$].angiotensin AT_{1A}, [$S^{109}A$].angiotensin AT_{1A}, [$N^{111}A$].angiotensin AT_{1A} and [$S^{115}A$].angiotensin AT_{1A} (Monnot et al., 1996). Expression plasmids containing wild-type and mutated angiotensin II receptors were transiently transfected into African green monkey SV40-transformed kidney cells (COS-7, ATCC# CRL1651) by the *O*-(diethylaminoethyl)-dextran chloroquine method and the cells were grown as described (Thibonnier et al., 1994). Binding studies or inositol phosphate measurements were done 48 to 72 h after transfection.

2.2.2. Radioligand binding assays

2.2.2.1. Saturation experiments. [Sar1]angiotensin II was labeled by the chloramine-T method and purified by high-performance liquid chromatography. Saturation binding experiments were carried out on intact cells for 45 min at 22°C using increasing concentrations of [^{125}I][Sar1]angiotensin II in the binding buffer (50 mM Tris–HCl (2-amino-2-hydroxymethylpropane-1-3-diol), 125 mM NaCl, 6.5 mM $MgCl_2$, 1 mM EDTA (ethylene diamine tetraacetic acid), 20 mM Hepes (4-(2-hydroxyethyl)1-piperazine-ethanesulphonic acid) and 1 mg/ml bovine serum albumin, pH 7.6) as described (Conchon et al., 1994). Nonspecific binding was determined in the presence of 1 μM [Sar1]angiotensin II.

2.2.2.2. Competition experiments. Competition binding experiments were performed on intact cells for 45 min at 22°C using [^{125}I][Sar1]angiotensin II (0.8–1 nM) and increasing concentrations (10^{-10} to 10^{-4} M) of L-162,313 (Conchon et al., 1994).

2.2.3. Phosphoinositide turnover

For the determination of the agonist properties of the nonpeptide compound, [^{3}H]inositol phosphate production in response to increasing concentrations (10^{-10} to 10^{-5} M) of angiotensin II or L-162,313 was measured as described previously (Torrens et al., 1989). Cells were labeled with 2 mCi/ml of *myo*-[^{3}H]inositol for 24 h and then incubated with agonist at 37°C for 30 min in the presence of 10 mM LiCl. After purification on a Dowex anion exchange resin (AG1-X8 resin, Bio-Rad), the total radiolabeled inositol phosphate fraction was measured. The determination of the antagonist properties of the nonpeptide compound was performed by the inositol phosphate accumulation in response to angiotensin II after pretreatment with L-162,313 (10^{-5} M) during 30 min.

2.3. Data analysis

Saturation and competition data were analyzed by a nonlinear least-squares curve fitting procedure, Ebda-Ligand (Elsevier-Biosoft, Cambridge, UK) (Munson and Rodbard, 1980).

Statistical analysis was performed by a paired Student's *t* test.

3. Results

The binding parameters of [^{125}I][Sar1]angiotensin II on rat angiotensin II receptor types and angiotensin AT_{1A} mutants individually expressed in COS-7 cells are shown in Table 1. The iodinated angiotensin II analog, [Sar1]angiotensin II, was selected as radioligand because of its high stability and its high affinity towards both angiotensin AT_1 and AT_2 receptors. Whereas no binding of iodinated [Sar1]angiotensin II was observed on untransfected COS-7 cells (data not shown), the transfected COS-7 cells expressed a very high density of wild-type or mutated angiotensin II receptors. Furthermore, all the receptors bound with the same affinity the iodinated [Sar1]angiotensin II. The wild-type and the mutated angiotensin II receptors had also a similar high affinity for angiotensin II (data not shown). This expression system allowed the study of the pharmacological and functional properties of L-162,313.

3.1. Binding affinity of L-162,313 for wild-type and angiotensin AT_{1A} mutated receptors

The binding of the nonpeptide compound L-162,313 on transiently expressed wild-type and mutant angiotensin II receptors was examined. Table 2 shows analysis of L-162,313 competition for [^{125}I][Sar1]angiotensin II on rat wild-type and mutated angiotensin II receptors. L-162,313 bound with the same affinities to the two angiotensin AT_1 receptor subtypes and the angiotensin AT_2 receptors. In contrast to the losartan, L-162,313 is not an AT_1-selective ligand. Furthermore, L-162,313 bound with reasonably high affinity to the rat angiotensin AT_1 and AT_2 receptors, but its affinity was much lower than those of the losartan to the angiotensin AT_1 receptors (2.36 nM for the angiotensin AT_{1A} receptor under the same conditions).

L-162,313 bound with different affinities to the angiotensin AT_{1A} receptor mutants. Three single-point mutants in the third transmembrane domain, [S^{107}A].AT_{1A}, [S^{109}A].AT_{1A} and [S^{115}A].AT_{1A} recognized L-162,313 with similar affinity as the wild-type angiotensin AT_{1A} receptor. In contrast, [D^{74}N].AT_{1A}, [D^{74}E].AT_{1A} and [S^{105}A].AT_{1A} showed a 2.5 or 3-fold significant lower affinity, whereas [N^{111}A].AT_{1A} showed a significant increase in affinity (3-fold).

Table 1
Binding parameters of [^{125}I][Sar^1]-angiotensin II in transfected COS-7 cells

	K_d (nM)	B_{max} (sites/cell)
AT_{1A}	0.31 ± 0.08	108 966 ± 13 765
AT_{1B}	0.18 ± 0.04	106 131 ± 12 295
AT_2	0.19 ± 0.07	81 368 ± 31 562
[D^{74}N].AT_{1A}	0.21 ± 0.06	58 133 ± 9967
[D^{74}E].AT_{1A}	0.24 ± 0.08	77 973 ± 29 604
[S^{105}A].AT_{1A}	0.34 ± 0.04	242 098 ± 22 243
[S^{107}A].AT_{1A}	0.26 ± 0.06	118 180 ± 16 454
[S^{109}A].AT_{1A}	0.24 ± 0.05	145 450 ± 44 777
[N^{111}A].AT_{1A}	0.18 ± 0.05	71 106 ± 8345
[S^{115}A].AT_{1A}	0.21 ± 0.08	32 834 ± 2412

Experiments, performed in duplicate, were repeated three to five times. The values reported are means ± S.E.

Table 2
Binding affinity of the nonpeptide L-162,313 for rat wild-type and mutated angiotensin AT receptors

	K_i (nM)
AT_{1A}	207 ± 20
AT_{1B}	226 ± 27
AT_2	276 ± 48
[D^{74}N].AT_{1A}	629 ± 39[b]
[D^{74}E].AT_{1A}	521 ± 10[a]
[S^{105}A].AT_{1A}	502 ± 41[a]
[S^{107}A].AT_{1A}	207 ± 38
[S^{109}A].AT_{1A}	189 ± 21
[N^{111}A].AT_{1A}	67 ± 8[a]
[S^{115}A].AT_{1A}	240 ± 52

Experiments, performed in duplicate, were repeated four to five times. The values reported are means ± S.E..
[a] $P < 0.05$.
[b] $P < 0.01$.

3.2. Agonist and antagonist activities of L-162,313 on wild-type and angiotensin AT_{1A} mutated receptors

The signal transduction properties of transiently expressed wild-type and mutant receptors were evaluated by measurements of inositol phosphate response induced by treatment with angiotensin II or L-162,313. The agonist effect of L-162,313 has been determined in COS-7 cells by comparison of the inositol phosphate accumulation in response to increasing concentrations of L-162,313 to that observed in response to a concentration of angiotensin II producing a maximal effect and considered as 100%. The full agonist effect of AngII has been determined in parallel in COS-7 cells resulting from the same transfection. Fig. 1 shows the partial agonist effect of L-162,313, compared to the full agonist effect of angiotensin II, in COS-7 cells expressing wild-type angiotensin AT_{1A} receptor or [S^{105}A].AT_{1A} or [N^{111}A].AT_{1A} mutants. L-162,313 increased inositol phosphate accumulation in a dose-dependent manner in transfected COS-7 cells with the rat angiotensin AT_{1A} (Fig. 1) or AT_{1B} receptors (data not shown). As expected, no response to increasing amounts of L-162,313 was observed on AT_2-transfected or untransfected COS-7 cells (data not shown). The maximal response to stimulation with L-162,313 was 3 to 5-fold lower than that observed with angiotensin II on the same transfected cells,

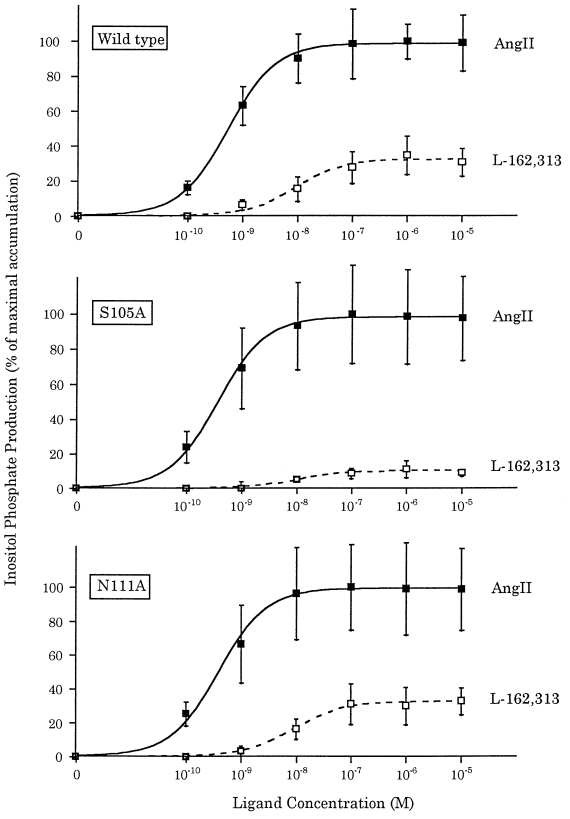

Fig. 1. Stimulation of inositol phosphate production by angiotensin II (AngII) or L-162,313 in transfected COS cells. The inositol phosphate production has been determined in COS cells expressing wild-type angiotensin AT_{1A} or mutated receptors ([S^{105}A].AT_{1A} and [N^{111}A].AT_{1A}) in response to increasing amounts of angiotensin II alone (■) or L-162,313 alone (□). Inositol phosphate accumulation is expressed as % of the maximal level reached in response to angiotensin II.

expressing either angiotensin AT_{1A} or AT_{1B} receptors (Table 3). The half-maximal response of the angiotensin AT_{1A} and AT_{1B} receptor subtypes was obtained with L-162,313 concentrations (EC_{50}) of 21 nM and 44 nM, respectively. L-162,313 is then an AT_1 partial agonist.

The potential antagonist properties of the L-162,313 were studied in the same expression system: the compound inhibited angiotensin II induced inositol phosphate accumulation by dramatically reducing the maximal response, in a dose-dependent manner. Thus, setting the maximal response to angiotensin II alone of the angiotensin AT_{1A} or AT_{1B} receptors as 100%, pretreatment with L-162,313 (10^{-5} M) reduced the maximal responses of angiotensin AT_{1A} and AT_{1B} receptors to angiotensin II to 63.7 and 46.2%, respectively (Table 3). In view of these reductions of these maximal responses, L-162,313 can be considered as an insurmountable antagonist.

The coupling efficiency of the seven single-point mutants of the angiotensin AT_{1A} receptor in response to L-162,313 has been studied. COS-7 cells expressing [D^{74}N].AT_{1A}, [D^{74}E].AT_{1A} or [S^{115}A].AT_{1A} mutants did not show any inositol phosphate accumulation in response to L-162,313 (Table 3), whereas a dose-dependent stimulation of inositol phosphate production was observed in cells expressing [S^{105}A].AT_{1A}, [S^{107}A].AT_{1A}, [S^{109}A].AT_{1A} or [N^{111}A].AT_{1A} mutants (Fig. 1 and Table 3). However, some variations were observed in the mutant responses to L-162,313. The inositol phosphate production of [S^{107}A].AT_{1A} and [N^{111}A].AT_{1A} was similar to that of the wild-type AT_{1A} for both the maximal response and the EC_{50} (37 and 11 nM, respectively). A significant antagonist effect of L-162,313 was also observed with these two mutants (Table 3). In contrast, the maximal stimulation of inositol phosphate production by the [S^{105}A].AT_{1A} and [S^{109}A].AT_{1A} mutants was lower than that observed for the wild-type. The intensity of the maximal inositol phosphate response to agonist (E_{max}, see Table 3) being dependent on the number of binding sites present at the surface of the cells (B_{max}, see Table 1) (Gershengorn et al., 1994), the ratio E_{max}:B_{max} confirms the lower coupling efficiency of the [S^{105}A].AT_{1A} and [S^{109}A].AT_{1A} mutants in response to

Table 3
Agonist and antagonist activities of the L-162,313 for rat wild-type and mutated angiotensin AT_1 receptors

	Agonistic effect	Antagonistic effect
	Response to L-162,313 E_{max} (%)	Response to angiotensin II after pretreatment with L-162,313 E_{max} (%)
AT_{1A}	34.9	63.7
AT_{1B}	23.3	46.2
[D^{74}N].AT_{1A}	0	–
[D^{74}E].AT_{1A}	0	–
[S^{105}A].AT_{1A}	11.3	100
[S^{107}A].AT_{1A}	29.8	77.2
[S^{109}A].AT_{1A}	13.1	100
[N^{111}A].AT_{1A}	32.8	56.1
[S^{115}A].AT_{1A}	0	–

The agonistic effect of L-162,313 is determined for the maximal level reached in response to L-162,313 and expressed as % of the maximal level reached in response to angiotensin II.
The antagonistic effect is determined for the maximal level reached in response to angiotensin II after pretreatment with L-162,313 (10^{-5} M) and expressed as % of the maximal level reached in response to angiotensin II alone.

L-162,313 (E_{max}:B_{max} = 4.6 and 9.0% of agonist effect/10^5 cells, respectively) than for the wild-type (E_{max}:B_{max} = 32.0% of agonist effect/10^5 cells). However, the dose–response curves were not shifted to the right: the EC_{50} of the [S^{105}A].AT_{1A} and [S^{109}A].AT_{1A} were comparable to the EC_{50} of the wild-type (12 nM and 47 nM, respectively).

4. Discussion

In the present study, we have examined the binding and the activity of the nonpeptide compound L-162,313 on the rat angiotensin AT_{1A}, AT_{1B} and AT_2 wild-type receptors, and on seven single-point mutants in the second and third transmembrane domains of the angiotensin AT_{1A} receptor. In contrast to the losartan which is a very potent AT_1-selective nonpeptide compound, L-162,313 is an AT_1 and AT_2 ligand. As all the other peptide and nonpeptide angiotensin II receptor ligands known today (Chiu et al., 1993), L-162,313 binds with the same affinity the two rat angiotensin receptor subtypes AT_{1A} and AT_{1B}. Furthermore, this nonpeptide compound is a partial agonist with both agonist and antagonist properties in vitro. However, this compound was found to be a full agonist in vivo (Kivlighn et al., 1995), whereas it increased blood pressure to the same degree as angiotensin II, it was shown to be a partial agonist in dipsogenic assays (Freidinger, 1993). L-162,313 seems then to be a partial agonist in vivo as in vitro, leading to a partial stimulation of the angiotensin AT_1 receptors (both AT_{1A} and AT_{1B}) but resulting in a full response on blood pressure increase.

The binding of L-162,313 is unaffected by three single-point mutations in the third transmembrane domain ([S^{107}A].AT_{1A}, [S^{109}A].AT_{1A} and [S^{115}A].AT_{1A}), which do not affect the binding of the nonpeptide antagonist losartan (Monnot et al., 1996). Moreover, its binding is affected by two mutations in the second and third transmembrane domains ([D^{74}N].AT_{1A} and [N^{111}A].AT_{1A}), which also affect the binding of losartan (Bihoreau et al., 1993; Monnot et al., 1996). These results suggest an overlap in the binding pocket of losartan and L-162,313 which have been described both mimicking the carboxy-terminus of angiotensin II in the binding to the angiotensin AT_1 receptor (Noda et al., 1995; Perlman et al., 1995). Furthermore, the third transmembrane domain of the angiotensin AT_1 receptor seems being critical for the nonpeptide recognition. In addition to the Asn^{111} involved in the binding of L-162,313, it has been recently reported that the Val^{108} is involved in the binding of biphenylimidazole and imidazoleacrylic acid ligands (Nirula et al., 1996), and the Ala^{104} plays a role in the binding of the nonpeptides L-162,782 and L-162,389 (Perlman et al., 1997). These results suggest that some polar residues of the second and third transmembrane domains are common structural determinants of nonpeptide recognition on the angiotensin AT_1 receptor.

However, the binding of L-162,313 is affected by one mutation in the third transmembrane domain ([S^{105}A].AT_{1A}), which do not affect the binding of losartan (Monnot et al., 1996). Then the two binding sites for nonpeptide antagonists and for nonpeptide partial agonists are not strictly identical, because some mutated residues selectively affect the binding of a single of the two ligands (Perlman et al., 1995; this report).

The partial agonist activity of L-162,313 and the full agonist activity of angiotensin II seem to involve a partially common activation mechanism of the angiotensin AT_1 receptor: Asp^{74} and Ser^{115} play a critical role for receptor activation in response to L-162,313 as well as it was showed for angiotensin II (Bihoreau et al., 1993; Monnot et al., 1996). The results of Noda et al. (1995) and the present studies indicate that the nonpeptide partial agonist L-162,313 and the peptide agonist angiotensin II may interact with common critical residues for receptor activation like Asp^{74}, Ser^{115} and His^{256} of the sixth transmembrane domain. However, the partial agonist activity seems to be due to the absence of some necessary interactions between the receptor and the agonist leading to the full activation of the angiotensin AT_1 receptor, like the interaction with the Asp^{281} side chain (at the junction of the third extracellular loop and the seventh transmembrane domain), which interacts with angiotensin II, but which does not interact with L-162,313 (Feng et al., 1995). L-162,313 seems to interact with other residues like Ser^{105} or Ser^{109}, which are not critical for the AT_{1A} activation in response to angiotensin II, but whose substitution leads to less efficient agonist and antagonist responses to L-162,313. These two residues seem then specifically involved in the conformational changes of the angiotensin AT_1 receptor, following the binding of the L-162,313. These results suggest that two polar residues of the second and third transmembrane domains (Asp^{74} and Ser^{115}) are common structural determinants involved in the mechanism of activation of the angiotensin AT_1 receptor by both peptide and non-peptide agonists, whereas two other polar residues of the third transmembrane domain (Ser^{105} and Ser^{109}) seem specifically involved in the agonistic and antagonistic activities of L-162,313.

In summary, the study of the pharmacological and functional properties of the nonpeptide compound L-162,313 have shown that this compound binds with the same affinity to the rat angiotensin II AT_{1A}, AT_{1B} and AT_2 receptors. Furthermore, in contrast to the nonpeptide AT_1 full antagonist losartan, this biphenylimidazole derivative is a partial agonist and shows some antagonist effect. The study of seven single-point angiotensin AT_{1A} receptor mutants indicate that L-162,313 and losartan have two distinct but partly overlapping binding sites in the hydrophobic pocket of the angiotensin AT_1 receptor (Nirula et al., 1996; Perlman et al., 1995, 1997). Finally, the partial agonist activity of L-162,313 and the full agonist activity of angiotensin II involve some common and some

different residues, which may suggest a partially common activation mechanism of the angiotensin AT_1 receptor (Noda et al., 1995; this report).

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