British Journal of Pharmacology (2000) 130, 1263–1268

www.nature.com/bjp

Mutational analysis of the interaction of the N- and C-terminal ends of angiotensin II with the rat AT_{1A} receptor

[1,2,3]Claudio M. Costa-Neto, [1]Ayumi A. Miyakawa, [1]Laerte Oliveira, [2]Siv A. Hjorth, [2]Thue W. Schwartz & *,[1]Antonio C.M. Paiva

[1]Department of Biophysics, Escola Paulista de Medicina, Federal University of São Paulo, 04023-062 São Paulo, SP, Brazil and [2]Laboratory for Molecular Pharmacology, Panun Institute, University of Copenhagen, Copenhagen, Denmark

1 The role of different residues of the rat AT_{1A} receptor in the interaction with the N- and C-terminal ends of angiotensin II (AngII) was studied by determining ligand binding and production of inositol phosphates (IP) in COS-7 cells transiently expressing the following AT_{1A} mutants: T88H, Y92H, G196I, G196W and D278E.

2 G196W and G196I retained significant binding and IP-production properties, indicating that bulky substituents in position 196 did not affect the interaction of AngII's C-terminal carboxyl with Lys^{199} located three residues below.

3 Although the T88A mutation did not affect binding, the T88H mutant had greatly decreased affinity for AngII, suggesting that substitution of Thr^{88} by His might hinder binding through an indirect effect.

4 The Y92H mutation caused loss of affinity for AngII that was much less pronounced than that reported for Y92A, indicating that His in that position can fulfil part of the requirements for binding.

5 Replacing Asp^{278} by Glu caused a much smaller reduction in affinity than replacing it by Ala, indicating the importance of Asp's β-carboxyl group for AngII binding.

6 Mutations in residues Thr^{88}, Tyr^{92} and Asp^{278} greatly reduced affinity for AngII but not for Sar^1 Leu^8-AngII, suggesting unfavourable interactions between these residues and AngII's aspartic acid side-chain or N-terminal amino group, which might account for the proposed role of the N-terminal amino group of AngII in the agonist-induced desensitization (tachyphylaxis) of smooth muscles.

British Journal of Pharmacology (2000) **130,** 1263–1268

Keywords: Angiotensin II; AT_1 receptor; G-protein; site-directed mutagenesis

Abbreviations: AngII, angiotensin II; AT_1 and AT_2, angiotensin type 1 and type 2 receptors; IP, inositol phosphates; TM, transmembrane segment

Introduction

Angiotensin II (AngII) receptors have been cloned from several species, including rat (Murphy *et al*., 1991). According to their selectivity for antagonists, two AngII receptor subtypes are distinguished: AT_1 (including isoforms AT_{1A} and AT_{1B}), and AT_2; AT_1 being responsible for the most important actions of AngII (Timmermans *et al*., 1993).

The AT_1 receptor belongs to the rhodopsin-like G-protein-coupled receptor family (GPCR) and has the characteristic seven transmembrane domain structure typical of such proteins (Figure 1). Binding of the peptide agonist involves residues situated in the extracellular domain of the receptor, mainly in the N-terminal domain and in the first and third extracellular loops (Hjorth *et al*., 1994; Feng *et al*., 1995), whereas residues located in the transmembrane region and intracelluar loops are associated with the triggering of the response (Bihoreau *et al*., 1993; Marie *et al*., 1994; Han *et al*., 1998) and G-protein activation (Oliveira *et al*., 1994; Scheer *et al*., 1996; Fanelli *et al*., 1998; Perlman *et al*., 1997).

When Tyr^{92}, in the first extracellular loop, was mutated to Ala, AngII binding was greatly affected, but no significant loss of affinity was observed when Thr^{88} and Met^{90}, few residues below, were both changed to Ala in a combined mutation (Hjorth *et al*., 1994). Similar mutation of Asp residues located in the same face of a putative helical extension of the seventh transmembrane segment (TM-VII) also showed their importance for binding AngII. Asp^{281} was suggested to interact with the Arg^2 of the peptide ligand (Feng *et al*., 1995), although Asp^{278} appears to be a more important contact point, since the mutation on that residue caused a decrease of affinity for AngII that was 50 fold that observed with the same mutation on Asp^{281} (Hjorth *et al*., 1994). It has also been proposed that Lys^{199}, positioned a few turns deep in TM-V, may be involved in an ionic interaction with the C-terminal caboxyl of the AngII molecule (Yamano *et al*., 1995; Noda *et al*., 1995).

Most of the mutational analysis of binding sites that have been done in seven transmembrane receptors, as well as in other proteins, used the Ala scan approach (Schwartz, 1994), but in cases where this generates false negative results the use of steric hindrance mutants should be useful for addressing the importance of a region of the receptor for ligand binding (Holst *et al*., 1998). Therefore, we have used this approach to investigate some residues which might participate or interfere in the receptor's interactions with the C- and N-terminal ends of the AngII molecule. To explore the possible interaction between the C-terminal carboxyl of AngII and the Lys^{199} side-chain, Gly^{196} (positioned three residues above on an helical turn) was mutated to Ile and to Trp, two hydrophobic and bulky residues that might cause a steric hindrance effect. In the region proposed to be involved in the interaction with AngII's N-terminus, we have mutated either Thr^{88} or Tyr^{92} to His.

*Author for correspondence; E-mail: acmpaiva@biofis.epm.br
[3]Current address: UNOESTE, Campus I, 19050-900 Presidente Prudente, SP, Brazil.

Regarding Tyr^{92}, the previously reported Y92A mutation dramatically affected the receptor's affinity for AngII (Hjorth *et al*., 1994) and mutation of this residue to His, for the same reasons described above, might provide more information on the participation of that residue in ligand binding. In the same region, but in the third extracellular loop close to TM-VII, Asp^{278}, which was previously mutated to Ala (Hjorth *et al*., 1994), was now mutated to Glu, a very conservative mutation, in order to analyse the importance of an acidic side-chain of the right size in that position.

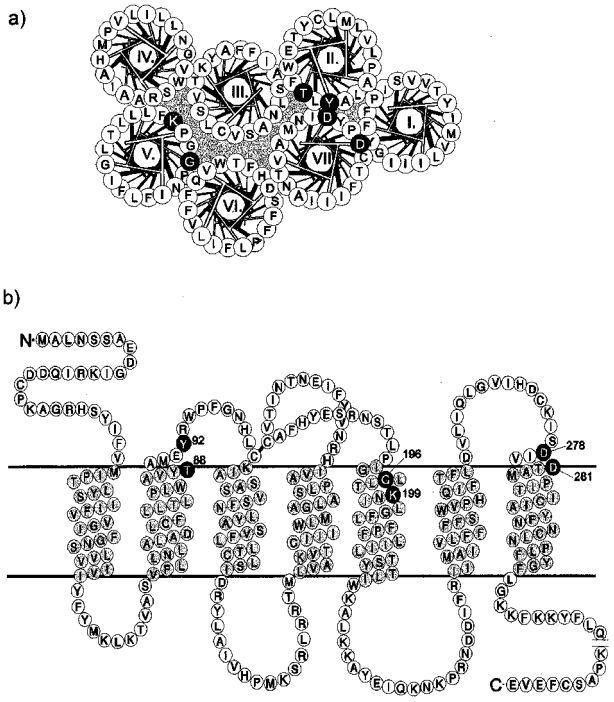

Figure 1 Helical wheel (a) and serpentine diagram (b) of the rat AT_1 receptor, indicating the putative transmembrane segments TM-I to TM-VII. The helical wheel scheme shows a top view of the helices disposed in anti-clockwise orientation, and with TM-III positioned as the central one. Residues that were analysed in this study are shown in white on black. Lys^{199} and Asp^{281}, discussed in the text, are shown in white on grey.

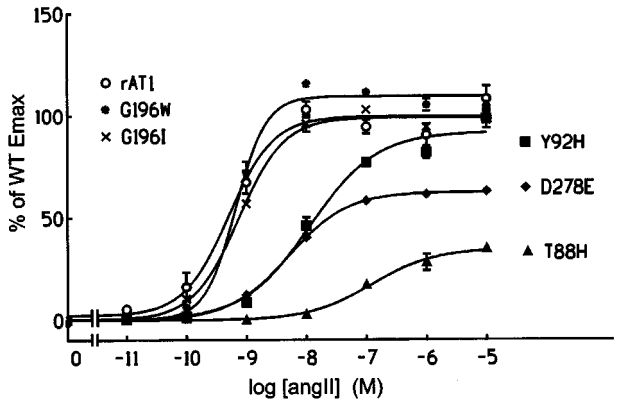

Figure 2 Concentration-response curves for the effect of AngII on IP turnover in transiently transfected COS-7 cells expressing wild type and mutant AT_1 receptors. The phosphatidylinositol turnover is expressed as a percentage of the E_{max} values obtained for the wild type receptor.

Methods

Materials

AngII and the peptide antagonist Sar^1,Leu^8-AngII were purchased from Peninsula Laboratories (St. Helens, Merseyside, U.K.). The non-peptide antagonist L-158,809 and the radiolabelled non-peptide antagonist ^{125}I-L735,286 were kind gifts from Dr William J. Greenlee (Merk Research Laboratories, Rahway, NJ, U.S.A.). Monoiodinated ^{125}I-Sar^1,Leu^8-AngII and ^{125}I-AngII were prepared by the IODO-GEN method as previously described (Sheikh *et al*., 1989) and purified by reverse-phase HPLC using a gradient of acetonitrile ranging from 19–27%. Materials and media for cell culture were obtained from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.).

Receptor mutagenesis

The rat AT_{1A} receptor cDNA (Murphy *et al*., 1991), generously provided by Dr T.J. Murphy (Emory University, Atlanta, GA, U.S.A.), was used to generate a 'cassette' gene, as previously described (Hjorth *et al*., 1994). Mutations were introduced by the PCR overlap extension technique (Horton *et al*., 1989). The PCR fragments were digested with the appropriate restriction enzymes and subsequently inserted into the likewise digested expression vector pTEJ8 (Johansen *et al*., 1990). *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.) was used for the PCR reactions under conditions recommended by the manufacturer. Temperature cycling consisted of 30–35 cycles at 94°C for 1 min, 45–50°C 1 min, 72°C 1 min. All receptor constructs were initially identified by the presence of a diagnostic restriction site, and subsequently verified by sequencing.

Cell culture and transfections

Expression plasmids containing wild type or mutated AT_1 receptors were transiently transfected into COS-7 cells by a calcium phosphate precipitation method as described before (Gether *et al*., 1993).

Inositol phosphate (IP) turnover

$0.3–0.4 \times 10^6$ cells expressing the wild type rat AT_1 receptor or mutants were cultivated for 24 h in inositol-free medium (1885 Dulbecco with NaH_2CO_3 supplemented with 10% foetal calf serum, 2 mM glutamine and 0.1 mg ml^{-1} gentamicin) in 6- or 12-well plates, each well containing 5 μCi ^{3}H-myoinositol (Amersham, Buckinghamshire, U.K.). The cells were washed twice with buffer (in mM): HEPES 20, NaCl 140, $MgSO_4$ 10, $CaCl_2$ 1, glucose 10, pH 7.4, and subsequently incubated for 5 min with the same buffer including 10 mM LiCl. AngII dose-response experiments were performed with 1-h incubation time. The reaction was terminated by the addition of 0.5 ml 10% perchloric acid and the precipitated cellular proteins were removed by centrifugation. The supernatants were collected and neutralized with 200 μl of a buffer solution (4.8 M KOH, 67.5 M HEPES) and incubated for 30 min with 2 ml of water and 0.5 ml of AG1-X8 anion-exchanger resin (Bio-Rad, Richmond, CA, U.S.A.) (Berridge *et al*., 1983). The resin was washed three times with 5 mM myoinositol and the ^{3}H-inositol phosphates were eluted by adding 1 ml of 1.0 M ammonium formate in 0.1 M formic acid. The data were analysed by nonlinear regression analysis using INPLOT 4.0 (Graph-Pad Software, San Diego, CA, U.S.A.).

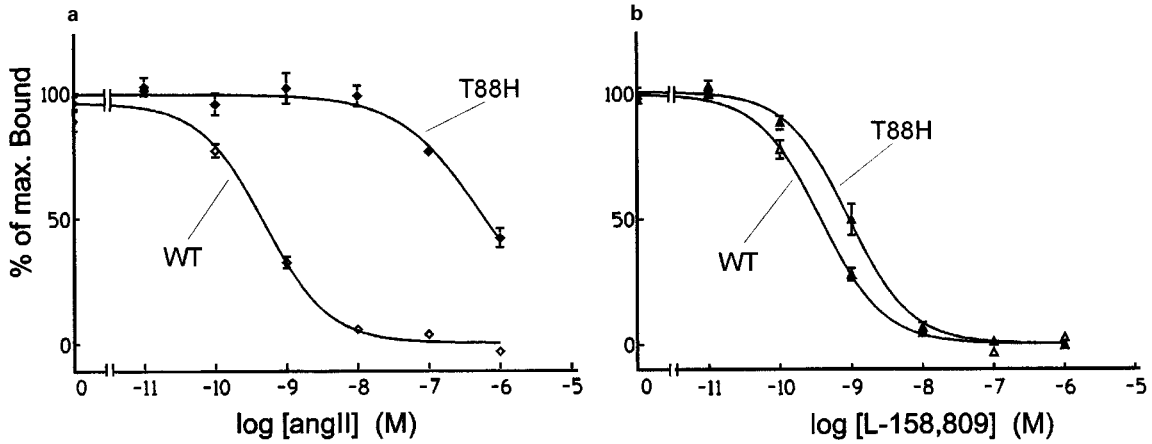

Figure 3 Competition binding profiles for AngII (a) and for the non-peptide antagonist L-159,809 (b) in cells transfected with the wild type (WT) and with the T88H mutant of the rat AT_1 receptor. Data are expressed as percentages of the maximum specific binding of the radioligand ^{125}I-L-735,286 (mean ± s.e.mean, $n = 4-12$).

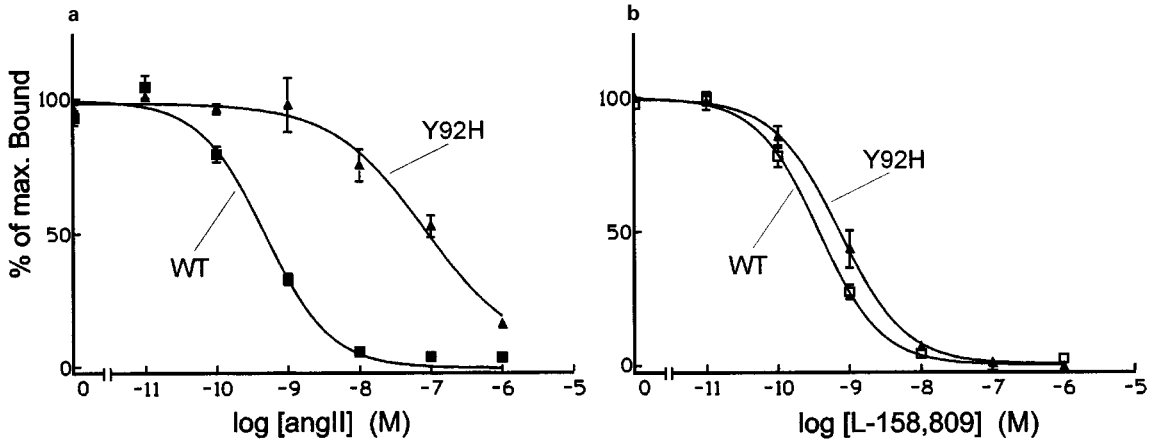

Figure 4 Competition binding profiles for AngII (a) and for the non-peptide antagonist L-159,809 (b) in cells transfected with the wild type (WT) and with the Y92H mutant of the rat AT_1 receptor. Data are expressed as percentages of the maximum specific binding of the radioligand ^{125}I-L-735,286 (mean ± s.e. mean, $n = 4-12$).

Whole cell binding experiments

Receptor binding assays were performed on whole COS-7 cells 48 h after transfection. Cells transfected with receptor expression plasmids were transferred to 12- or 24-well culture plates 24 h after transfection aiming at $0.2-1.5 \times 10^5$ cells per well, depending on the expression level of the plasmid used. Twenty-four hours following plating of the transfected cells, and immediately before the binding experiment, the cells were washed briefly in 25 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl, 5 mM $MgCl_2$ and 0.1% bovine serum albumin. Binding experiments were performed at 4°C and initiated by the addition of 50 pM ^{125}I-L735,286, or ^{125}I-Sar^1-Leu^8-AngII, or ^{125}I-AngII in the presence of varying amounts of unlabelled peptide or non-peptide ligand as a competitor in a 0.5–1.0 ml assay volume. The binding buffer consisted of 25 mM Tris-HCl, pH 7.4, including 5 mM $MgCl_2$, 0.1% bovine serum albumin, and 100 μg ml^{-1} bacitracin (Sigma Chemical Co., St. Louis, MO, U.S.A.). All measurements were done in triplicate, and the data were analysed by nonlinear regression analysis using INPLOT 4.0 (Graph-Pad Software, San Diego, CA, U.S.A.).

Results and discussion

Tables 1 and 2 summarize the effects of the mutations on the AT_1 receptor as determined by competition binding experiments using either the non-peptide compound ^{125}I-L-735,286 or ^{125}I-AngII (homologous competition) as radioligands. ^{125}I-L-735,286 was used more extensively as radioligand because the mutations studied were located in regions supposedly involved in interactions with the natural peptide ligand AngII, and it is known that peptide and non-peptide ligands bind to different sites in the receptor. The mutants that presented greater loss of affinity for ^{125}I-L-735,286 (F(mut) > 10) were also tested in competition assays with the peptide antagonist ^{125}I-Sar^1,Leu^8-AngII. Table 1 shows that the mutations that affected AngII binding did not significantly reduce the receptor's affinity for the non-peptide antagonist L-158,809. However, the sole use of a non-peptide as a radioligand might lead to an overestimation of the observed loss of affinity in some of the mutants studied. For that reason, we also used two other radioligands: the peptide antagonist ^{125}I-Sar^1,Leu^8-AngII (Table 1) and the peptide agonist ^{125}I-AngII (used for B_{max} calculation, Table 2).

Table 1 Binding affinity of AngII, Sar[1], Leu[8]-Ang II, and L-158,809 for wild type and mutant rat AT_1 receptors

	^{125}I-L-735,286							^{125}I-Sar[1], Leu[8]-Ang II					
	Angiotensin II			Sar[1], Leu[8]-Ang II			L-158,809	Angiotensin II			Sar[1], Leu[8]-Ang II		L-158,809	
	IC_{50} (nM)	(n)	F_{mut}	IC_{50} (nM)	(n)	F_{mut}	IC_{50} (nM)	(n)	F_{mut}	IC_{50} (nM)	(n)	F_{mut}	IC_{50} (nM)	(n)	F_{mut}
wt rAT_1	0.49±0.05	(9)	–	0.19±0.02	(9)	–	0.41±0.05	(12)	–	0.58±0.09	(5)	–	0.30±0.05	(5)	–	0.55±0.04	(7)	–
T88H	653±113	(4)	1333	3.10±0.39	(4)	16	0.90±0.16	(5)	2.2	505±76	(3)	871	1.70±0.33	(3)	6	1.24±0.07	(3)	2.3
Y92H	95.4±29	(3)	195	6.90±1.3	(3)	36	0.83±0.20	(5)	2.0	66.8±20	(3)	115	2.30±0.23	(3)	8	1.28±0.25	(3)	2.3
Y92A[a]			3110[a]			25			5.9		ND			ND			ND	
G196I	0.92±0.15	(3)	1.9	0.26±0.03	(3)	1.4	0.57±0.08	(3)	1.4		ND			ND			ND	
G196W	2.60±0.51	(4)	5.3	0.25±0.08	(4)	1.3	1.80±0.27	(4)	4.4		ND			ND			ND	
D278A[a]			813			71			0.5		ND			ND			ND	
D278E	36.5±6.7	(5)	75	0.20±0.04	(4)	1.1	0.59±0.16	(5)	1.4	14.3±6.0	(3)	25	0.39±0.04	(3)	1.3	0.63±0.39	(3)	1.1

Binding affinities were determined in competition binding experiments using ^{125}I-L-735,286 and ^{125}I-Sar[1], Leu[8]-Ang II as radioligands. Values are means±s.e.mean and the number of independent experiments done in triplicate is shown in parentheses. F_{mut} is the ratio of the IC_{50} values for the mutant and wild type receptors. ND, not determined. [a]Data published before (Hjorth *et al.*, 1994), included here for comparison.

Table 2 IC_{50} and B_{max} values for AngII binding to wild type and mutants of the rat AT_1 receptor

Receptor	IC_{50} (nM)	(n)	F_{mut}	B_{max} (fmol 10^{-3} cells)
wt rAT_1	0.31±0.03	(12)	–	61±9.8
T88H		NB		–
Y92H	1.57±0.22	(6)	5.1	20±3.7
G196I	0.62±0.27	(3)	2.0	81±16.7
G196W	0.45±0.09	(3)	1.5	73±11.8
D278E	0.92±0.24	(7)	3.0	12±4.9

IC_{50} and B_{max} values were determined in competition binding experiments using ^{125}I-AngII as radioligand. Values are means±s.e.mean, and the number of independent experiments done in triplicate is shown in parentheses. The effect of the mutations is expressed by a 'mutation factor', F_{mut}, defined as the ratio between IC_{50} (mutant) and IC_{50} (wild type). NB, no detectable binding.

Table 3 Effect of AngII on IP accumulation in COS-7 cells transfected with wild type and mutant AT_1 receptors

Receptor	EC_{50} (nM)	(n)	F_{mut}	E_{max} (pmol 10^{-5} cells h^{-1})
wt rAT_1	0.51±0.1	(5)	–	0.35±0.04
T88H	198±78	(3)	421	0.13±0.04
Y92H	11.3±2.5	(5)	24	0.32±0.06
G196I	0.74±0.04	(4)	1.6	0.35±0.06
G196W	0.63±0.07	(3)	1.3	0.39±0.05
D278E	5.05±0.4	(4)	11	0.22±0.10

EC_{50}, and E_{max} values were obtained in IP assays using AngII as the agonist. Values are means±s.e.mean, and the number of independent experiments done in triplicate is shown in parentheses.

The functional state of the mutants was assessed by the inositol phosphate accumulation in response to AngII and the results are presented in Table 3.

Gly^{196}

In spite of the potential steric consequences on Lys^{199}, replacing Gly^{196} residue by Ile or by Trp affected very little the receptor's affinity for AngII as determined by competition experiments using ^{125}I-L-735,286 as radioligand (Table 1). The wild type receptor and the G1961 or G196 W mutants presented similar IC_{50} values for binding AngII and Sar[1],Leu[8]-AngII, as well as the non-peptide antagonist L-158,809. Homologous competition binding experiments using ^{125}I-AngII as radioligand were also performed (Table 2), and the B_{max} values found for G1961 and for G196 W were similar to those obtained for the wild type receptor. The results of the IP accumulation assays are shown in Figure 2. No significant differences were also found between the EC_{50} and E_{max} values obtained for the two mutants and for the wild type receptor (Table 3). These unexpected results might be due to one of the following situations: (a) Gly^{196} is not on a transmembrane region, i.e. its position is not constrained above Lys^{199}; (b) the two residues are in a transmembrane region, but the Lys^{199} side-chain could be pointing toward the extracellular surface of the receptor, in a snorkel effect, allowing AngII's C-terminal end to interact without having to penetrate the transmembrane domain of the receptor; (c) the receptor pocket formed on the interfaces of TM-III, TM-V, and TM-VII may have a higher degree of freedom, allowing the peptide's C-terminus to reach the Lys^{199} side-chain even in the presence of the bulky substituents introduced in the G1961 and G196W mutants.

Thr^{88}

As expected if Thr^{88} were in fact located in an important region, the T88H mutation decreased the affinity constant for AngII by more than 1000 fold (Figure 3a). The affinity for Sar^1, Leu^8-AngII and for L-158,809 was much less affected, as illustrated in Figure 3b for the case of L-158,809. Table 1 shows that the loss of affinity of the T88H mutant for Sar^1, Leu^8-AngII and L-158,809 ranged from 2.2–16 fold when either ^{125}I-L-735,286 or ^{125}I-Sar^1, Leu^8-AngII was used as the radioligand. As a result of its very low affinity for AngII the mutant T88H was not able to bind ^{125}I–AngII, and therefore no B_{max} value could be obtained to be included in Table 2. Nevertheless, this mutant was still able to stimulate IP turnover, although with very high EC_{50} and low E_{max} values (Figure 2 and Table 3).

Since replacement of Thr^{88} by alanine did not affect the binding of AngII (Hjorth *et al*., 1994), it is conceivable that substitution by His might hinder AngII binding through an indirect effect. Histidine is an amino acid that displays both hydrophobic and polar characteristics, with a partial positive charge at neutral pH. A mutation of Thr^{88} to His might affect AngII binding through an ionic interaction with acidic residues (such as Asp^{278} or Asp^{281}) supposed to be directly involved in AngII's binding, or by disrupting a cluster of residues involved on the formation of a binding site such as Tyr^{92}, Asp^{278} or Asp^{281} (Hjorth *et al*., 1994; Feng *et al*., 1995).

Tyr^{92}

Previous mutation of this residue to Ala (Hjorth *et al*., 1994) caused a very large loss of affinity for AngII in competition binding experiments using the non-peptide antagonist ^{125}I-L-735,286 as radioligand. In the case of the Y92H mutant, when ^{125}I-L-735,286 or ^{125}I-Sar^1,Leu^8-AngII were used as radioligands (Table 1), the affinity for AngII was greatly decreased with regard to that of the wild type receptor (Figure 4a), but not as much as occurred with the Y92A mutant. Also similarly to what was observed when Tyr^{92} was replaced by Ala, the affinity of the Y92H mutant for the antagonists Sar^1, Leu^8-AngII and L-158,809 (Figure 4b) was not much affected as compared to that of the wild type receptor. In homologous binding experiments using ^{125}I-AngII as the radioligand, only a 5.1 fold reduction in the affinity for AngII was observed, while the calculated B_{max} value was significantly lower than that found for the wild type receptor (Table 2).

Regarding the IP accumulation response (Figure 2 and Table 3), AngII stimulated the Y92H mutant with an EC_{50} value 24 times higher than that of the wild type receptor. The E_{max} value, however was not different from that obtained for the wild type receptor. Therefore, His at position 92 still retains some of the contribution of the Tyr residue for ligand binding by AT_1. The imidazole and phenolic moieties present in the His and Tyr side-chains, respectively, have some characteristics not present in Ala, such as their aromatic character, their partly hydrophobic partly polar properties, their ability as hydrogen bond makers, as well as their bulk. Which of these characteristics might be important for the role of Tyr^{92} in AT_1 ligand binding is not possible to say with the present results.

Asp^{278}

Using ^{125}I-L-735,286 as the radioligand, the binding constant of the D278E mutant for AngII was decreased 75 fold, but that for Sar^1,Leu^8-AngII was basically not affected (Table 1). When ^{125}I-AngII was used as the radioligand, the competition binding assay revealed little change in the affinity of D278E for AngII, but the B_{max} value obtained was significantly decreased (Table 2). In cells transfected with this mutant, the dose response-curve for the effect of AngII on IP accumulation was shifted to the right, with a 10 fold increase in the EC_{50} value, and E_{max} was decreased as compared to the wild type (Figure 2 and Table 3). This indicates that the carboxyl group of the side-chain, also present in Glu, is mostly responsible for the contribution of Asp^{278} to the affinity of AT_1 for AngII. Yet, it shows the important participation of Asp^{278} in the interaction with AngII, since in the D278E mutant a single amino acid residue in the entire receptor molecule had its side-chain modified with the 'insertion' of an extra methylene group.

It is interesting to note that the loss of AngII binding observed in mutants T88H, Y92H and D278E was not accompanied by a similar loss of affinity for the peptide analogue Sar^1, Leu^8-AngII. This suggests that mutations in those residues might allow interactions between receptor and ligand that would be unfavourable to the binding of AngII, but not to that of Sar^1,Leu^8-AngII. This unfavourable interaction would involve either the aspartic acid side-chain or the primary N-terminal amino group of AngII, which are absent in Sar^1,Leu^8-AngII, and might account for the proposed role of the N-terminal amino group of AngII in the agonist-induced desensitization (tachyphylaxis) observed in smooth muscle (Oshiro *et al*., 1989).

This study was supported by grants from the São Paulo State Research Foundation (FAPESP), the Brazilian National Research Council (CNPq), the Danish Medical Research Council and the Novo Nordisk Foundation.

References

BERRIDGE, M.J., DAWSON, M.C., DOWNES, C.P., HESLOP, J.P. & IRVINE, R.F. (1983). Changes in the level of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.,* **212,** 473–482.

BIHOREAU, C., MONNOT, C., DAVIES, E., TEUTSCH, B., BERNSTEIN, K.E., CORVOL, P. & CLAUSER, E. (1993). Mutation of Asp74 of the rat angiotensin II receptor confers changes in antagonist affinities and abolishes G-protein coupling. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 5133–5137.

FANELLI, F., MENZIANI, C., SCHEER, A., COTECCHIA, S. & DE BENEDETTI, P.G. (1998). Ab initio modeling and molecular dynamics simulation of the alpha 1b-adrenergic receptor activation. *Methods,* **14,** 302–317.

FENG, Y., NODA, K., SAAD, Y., LIU, X., HUSAIN, A. & KARNIK, S.S. (1995). The docking of Arg^2 of angiotensin II with Asp^{281} of AT_1 receptor is essential for full agonism. *J. Biol. Chem.,* **170,** 12846–12850.

GETHER, U., JOHANSEN, T.E. & SCHWARTZ, T.W. (1993). Chimeric NK_1 (substance P)/NK_3 (neurokinin B) receptors - Identification of domains determining the binding specificity of tachykinin. *J. Biol. Chem.,* **268,** 7893–7898.

HAN, H.M.C.B., SHIMUTA, S.I., KANASHIRO, C.A., OLIVEIRA, L., HAN, S.W. & PAIVA, A.C.M. (1998). Residues Val^{254}, His^{256}, and Phe^{259} of the angiotensin II AT_1 receptor are not involved in ligand binding but participate in signal transduction. *Mol. Endocrinol.,* **12,** 810–814.

HJORTH, A.S., SCHAMBYE, H.T., GREENLEE, W.J. & SCHWARTZ, T.W. (1994). Identification of peptide binding residues in the extracellular domains of the AT_1 receptor. *J. Biol. Chem.,* **269,** 30953 – 30959.

HOLST, B., ZOFFMANN, S., ELLING, C.E., HJORTH, S.A. & SCHWARTZ, T.W. (1998). Steric hindrance mutagenesis versus alanine scan in mapping of ligand binding sites in the tachykinin NK1 receptor. *Mol. Pharmacol.,* **53,** 166 – 175.

HORTON, R.M., HUNT, H.D., HO, S.N., PULLEN, J.K. & PEASE, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene,* **77,** 61 – 68.

JOHANSEN, T.E., SCHOLER, M.S., TOLSTOY, S. & SCHWARTZ, T.W. (1990). Biosynthesis of peptide precursors and protease inhibitors using new constitutive and inducible eukariotic expression vectors. *FEBS Lett.,* **267,** 289 – 294.

MARIE, J., MAIGRET, B., JOSEPH, M., LARGUIER, R., NOUET, S., LOMBARD, C. & BONNAFOUS, J. (1994). Tyr^{292} in the seventh transmembrane domain of the AT_{1a} angiotensin II receptor is essential for its coupling to phospholipase C. *J. Biol. Chem.,* **269,** 20815 – 20818.

MURPHY, T.J., ALEXANDER, R.W., GRIENDLING, K.K., RUNGE, M.S. & BERNSTEIN, K.E. (1991). Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature,* **351,** 233 – 336.

NODA, K., SAAD, Y. & KARNIK, S.S. (1995). Interaction of Phe^{8} of angiotensin II with Lys^{199} and His^{256} of AT_1 receptor in agonist activation. *J. Biol. Chem.,* **270,** 28511 – 28514.

OLIVEIRA, L., PAIVA, A.C.M., SANDER, C. & VRIEND, G. (1994). A common step for signal transduction in G protein-coupled receptors. *Trends Pharmacol. Sci.,* **15,** 170 – 172.

OSHIRO, M.E.M., SHIMUTA, S.I., PAIVA, T.B. & PAIVA, A.C.M. (1989). Evidence for a regulatory site in the angiotensin II receptor of smooth muscle. *Eur. J. Pharmacol.,* **166,** 411 – 417.

PERLMAN, S., COSTA-NETO, C.M., MIYAKAWA, A.A., SCHAMBYE, H.T., HJORTH, A.S., PAIVA, A.C.M., RIVERO, R.A., GREENLEE, W.J. & SCHWARTZ, T.W. (1997). Dual agonistic and antagonistic property of non-peptide angiotensin AT_1 ligands - Susceptibility to receptor mutagenesis. *Mol. Pharmacol.,* **51,** 301 – 311.

SCHEER, A., FANELLI, F., COSTA, T., DE BENEDETTI, P.G. & COTECCHIA, S. (1996). Constitutively active mutants of the alfa 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *EMBO J.,* **15,** 3566 – 3578.

SCHWARTZ, T.W. (1994). Locating ligand-binding sites in 7TM receptors by protein engineering. *Curr. Opin. Biotech.,* **5,** 434 – 444.

SHEIKH, S.P., O'HARE, M.M.T., TORTORA, O. & SCHWARTZ, T.W. (1989). Binding of monoiodinated neuropeptide Y to hippocampal membranes and human neuroblstoma cell lines. *J. Biol. Chem.,* **264,** 6648 – 6654.

TIMMERMANS, P.B.M.W.M., WONG, P.G., CHIN, A.T., HERBLIN, W.F., BENFIELD, P., CARINI, D.J., LEE, R.J., WEXLER, R.R., SAYE, J.A.M. & SMITH, R.M. (1993). Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Ver.,* **45,** 205 – 251.

YAMANO, Y., OHYAMA, K., KIKYO, M., SANO, T., NAKAGOMI, Y., INOUE, Y., NAKAMURA, N., MORISHIMA, I., GUO, D.-F., HAMAKUBO, T. & INAGAMI, T. (1995). Mutagenesis and the molecular modeling of the rat angiotensin II receptor (AT1). *J. Biol. Chem.,* **270,** 14024 – 14030.

(Received November 1, 1999
Revised April 14, 2000
Accepted April 14, 2000)