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AMINO ACIDS OF THE THIRD TRANSMEMBRANE DOMAIN OF THE AT_{1A} ANGIOTENSIN II RECEPTOR ARE INVOLVED IN THE DIFFERENTIAL RECOGNITION OF PEPTIDE AND NONPEPTIDE LIGANDS

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SUMMARY: The differential role of amino acids of the third transmembrane domain on peptide and nonpeptide recognition by the AT_1 angiotensin II receptor has been evidenced. The mutation of Ser^{105} into alanine completely abolished peptide agonist and antagonist binding, while the binding of nonpeptide ligands, including the original radioligands [^{3}H] LF 7-0156 and [^{3}H] LF 8-0129, was more moderately affected. Reverse pharmacological changes, i.e., unchanged affinities for peptide agonists or antagonists and drastically reduced affinities for nonpeptide antagonists, were observed upon alanine replacement of Asn^{111}. These results confirm that the binding sites for peptide and nonpeptide molecules are not totally overlapping and delineate new amino acids as candidates for the selective receptor interaction with the two categories of ligands. Their integration in topographical studies is discussed.

The development of selective nonpeptide or pseudopeptide antagonists has allowed pharmacological discrimination of AT_1 and AT_2 angiotensin II (AII) receptor subtypes (1-3). While the known pressor effects of AII are mediated by the AT_1 receptor, the physiological role of the AT_2 receptor and the associated transduction mechanisms remain unclear. The cloning of the cDNAs encoding the AT_1 (4,5) and AT_2 (6,7) receptors reveal that they belong to the superfamily of seven transmembrane domain receptors.

Besides their potential use as antihypertensive agents, nonpeptide antagonists constitute appropriate tools for the structural analysis of receptors and dissection of the molecular events associated to the blockade of agonist-induced receptor activation. In this respect, a fundamental question is the extent of spatial overlapping between active conformations of peptide and nonpeptide pharmacological agents.

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Partial answers to this question have arosen from point mutagenesis or chimeric receptor construction experiments. Studies on neurokinin receptors have delineated epitopes (8-12) or single amino acids (11-15) that are differently involved in the binding of peptide and nonpeptide ligands. Similar conclusions were drawn for CCK-B/gastrin receptors (16,17), opiate receptors (19) and quite recently for the AT_1 angiotensin II receptor (20,21).

Previous biochemical data (22) and preliminary modeling studies (23) have suggested that the third transmembrane domain of the AT_{1A} receptor might play a role in angiotensin II recognition and the initial events leading to receptor activation. It prompted us to perform a systematic analysis of the role of the four polar residues Ser^{105}, Ser^{107}, Ser^{109} and Asn^{111} located within a double helix turn in the upper part of the third transmembrane domain, which might be involved in hydrogen bond receptor-ligand interactions. In the present work, we checked possible modifications of peptide and nonpeptide binding by site-directed mutagenesis of these residues as well as Lys^{102} residue, located at the interface between the third transmembrane domain and the extracellular medium, which might be responsible for ionic interaction with the two classes of ligands.

MATERIALS AND METHODS

Reagents: Sar^1-AII and [Sar^1-Ile^8] AII were purchased from Bachem (Bubendorf, Switzerland); Sar^1-AII was radioiodinated as previously described (24). Nonpeptide antagonists LF 7-0156, LF 8-0129 (25), DuP 753 and WL 19 were synthesized by Fournier Laboratories (Daix, France). CGP 42112A was provided by Drs M. De Gasparo and S. Bottari (Ciba-Geigy, Basel, Switzerland). [^{3}H] DuP 753 and myo-[2-^{3}H] inositol (23-40 Ci/mmole) were from NEN. The custom synthesis of [^{3}H] LF 7-0156 (4.6 Ci/mmole) (26) and [^{3}H] LF 8-0129 (20.2 Ci/mmole) were carried out by CEA (Saclay, France) and Isotopchim (Ganagobie, France), respectively.

Site-directed mutagenesis and expression :

The entire cDNA sequence of the rat AT_{1A} receptor cloned in the pECE vector (27) was excised with Hind III and Eco R1 to produce a 0.8 Kb fragment which was inserted into the polylinker of the M13mp19 vector, allowing obtention of single strand DNA. Single amino acid mutations were carried out using the oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.) as previously described (28). The DNA sequences of mutated receptors were confirmed by the dideoxynucleotide chain termination method (T7 sequencing kit, Pharmacia).
For routine pharmacological characterization, the mutants were transiently expressed in COS-7 cells using the pECE vector and minor modifications of the DEAE-dextran transfection method (29). Plasma membranes were prepared 3 days after cell transfection (culture medium: DMEM, 4, 5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin). When required, higher receptor transient expression levels associated to homogeneous expression per cell were obtained through electroporation and use of the eukaryotic expression vector pCMV (30). Inositol phosphate accumulation assays were carried out three days after cell transfection.

Alternatively, pharmacological characterization was performed on receptors stably expressed in CHO cells as previously described (28). The clones displaying the highest receptor densities were selected through radioligand binding carried out on intact cells. CHO cells stably expressing the wild-type rat AT_{1A} receptor were a generous gift from E. Clauser (Paris, France) and K. Bernstein (Atlanta, USA).

Binding assays:

Plasma membrane : Crude membranes from COS-7 cells transiently expressing the wild-type or mutated AT_{1A} receptors were prepared as previously described (28). Protein concentrations were measured according to Lowry (31).
[^{125}I]Sar^1-AII binding (10-15 µg membrane protein, 90 µl volume) and[^{3}H] DuP 753, [^{3}H] LF 7-0156 and [^{3}H] LF 8-0129 binding (45µg protein, 90 µl volume) were performed as in (27) and (25) respectively. Competition binding experiments were carried out using 1 nM [^{125}I]Sar^1-

AII and increasing concentrations of the various ligands as previously indicated for non peptide binding (26). K_i were calculated according to Cheng and Prusoff (32).

Intact cells : Transfected CHO cells grown in 12-well plates (about 5 x 10^5 cells/plate) were incubated for 4hours at 4°C with the various radioligands in the absence or the presence of a 100-fold excess of unlabeled Sar^1-AII (28).

Inositol phosphate assays :

COS-7 cells expressing the wild type or mutant AT_{1A} receptors were grown in 6-well tissue culture clusters and labelled for 24 hours with [2-^{3}H] inositol (1.5 ml/well, 1 µCi/ml) in DMEM medium without serum and unlabelled inositol. Pooled inositol phosphates were extracted and measured as previously described (28,33) after a 15 min Sar^1-AII stimulation.

RESULTS

The polar residues Ser ^{105}, Ser ^{107}, Ser^{109} and Asn^{111} were mutated into alanine, while Lys^{102} was mutated into methionine in order to create a possible cyanogen bromide cleavage site for further biochemical mapping experiments (22).

Most of the pharmacological properties of the wild type (WT) or mutant receptors were established through competition binding experiments using [^{125}I] Sar^1-AII as tracer ligand. The analysis of mutants displaying marked increase in K_i values as compared to the wild-type receptor was completed by direct binding experiments of the concerned ligands. This study was facilitated by the previous development of original tritiated nonpeptide antagonist completely devoid of non specific binding (26).

Competition binding experiments using [^{125}I] Sar^1-AII tracer ligand revealed that Ala replacement of Ser^{107} and Ser^{109} did not significantly affect the K_i values for the peptide agonist Sar^1-AII, the peptide antagonist [Sar^1, Ile^8] AII or the AT_1 specific non peptide antagonist DuP 753 (Table 1).

The affinities of all tested peptide and nonpeptide ligands were reduced in the K102M mutant as compared to the wild-type receptor (Table 1). The increase in K_i values was more important for DuP 753 (almost two orders of magnitude) than for peptide ligands (7-fold and 4-fold increase for the agonist Sar^1-AII and antagonist [Sar^1, Ile^8] AII, respectively).

No binding of the peptide agonist [^{125}I] Sar^1-AII or antagonist [^{125}I] [Sar^1, Ile^8] AII could be detected at concentrations up to 100 nM on the S105A mutant receptor stably expressed in CHO cells, at densities in the range 2-3 x 10^5 sites/cell (Figure 1); the possibility to select stable transfectant clones through the binding of [^{3}H] LF 7-0156 demonstrated that this lack of recognition was not due to a modified receptor expression but actually reflects a differential recognition of peptide and nonpeptide ligands. Indeed, it was possible to perform accurate determinations of binding parameters for two original nonpeptide antagonists [^{3}H] LF 7-0156 (K_d = 117±7 nM, n = 3) and [^{3}H] LF 8-0129 (K_d = 11.7±1.8 nM, n = 3). The extent of K_d increase for the S105A receptor as compared to the wild-type receptor did not exceed one order of magnitude for the high affinity ligand [^{3}H] LF 8-0129 (Figure 1). The binding parameters for [^{3}H] DuP 753 could not be accurately evaluated because of the previously mentioned higher non specific binding (26, 34, 35), which emphasizes the interest of [^{3}H] LF 7-0156 and [^{3}H] LF 8-0129 as original new tools for the evaluation of pharmacological properties of mutant receptors (26).

Table 1 : Compared binding affinities of peptide and nonpeptide ligands for the wild type and mutant AT_{1A} receptors expressed in COS-7 cells

Ligand	K_I (nM) Receptors				
	WT	K102M	S107A	S109A	N111A
Peptide agonist					
Sar^1-AII	1.8 ± 0.4	11.8 ± 1.5	1.0 ± 0.1	2.5 ± 0.5	1.9 ± 0.5
Peptide antagonist					
[Sar^1, Ile^8]-AII	2.4 ± 1.4	9.9 ± 0.6	2.5 ± 1.3	3.4 ± 0.6	5.2 ± 1.5
Nonpeptide antagonists					
DUP 753	7.7 ± 1.57	561 ± 95	6.1 ± 0.2	7.3 ± 2.7	1600 ± 458
CGP 42112A	2765 ± 1030	> 10 0000	2150 ± 250	3650 ± 1300	96.5 ± 5.0
WL-19	> 10 000	> 10 000	> 10 000	> 10 000	> 10 000
LF 7-0156	8.0 ± 2.1	nd	nd	nd	883 ± 306
LF 8-0129	4.5 ± 1.1	nd	nd	nd	406 ± 52

K_i values were calculated from competition binding experiments, carried out on membrane preparations from COS-7 cells transiently expressing the wild type (WT) or mutant AT_{1A} receptors, using [^{125}I]Sar^1-AII as tracer ligand, as described in "Materials and Methods". Data are expressed as mean ± SD of three experiments, each performed in triplicate (nd : not determined).

Reverse pharmacological changes, i.e. unchanged affinity for peptide ligands and drastically reduced affinities for nonpeptide antagonists were observed for the N111A mutant receptor. The binding of [^{125}I] Sar^1-AII to the AT_1 receptor transiently expressed in COS-7 cells was not significantly affected by alanine replacement of the Asn^{111} residue (Figure 1). Competition binding experiments with [^{125}I] Sar^1-AII as tracer ligand also revealed unchanged characteristics for the binding of the peptide antagonist [Sar^1, Ile^8]-AII (Table 1). However, this mutation severly impaired the recognition of the nonpeptide ligands LF 7-0156, LF 8-0129 and DuP753, the corresponding K_i values being 100-200-fold increased as compared to their counterparts in the WT receptor (Table 1). It was confirmed by direct binding of the radioligands [^{3}H] LF 7-0156 and [^{3}H] LF 8-0129 and [^{3}H] DuP 753 which displayed so low affinities for the N111A mutant receptor that accurate evaluation of their binding parameters was not possible (Figure 1D). At the opposite, the N111A mutant possessed an increase affinity for CGP 42112A (Table 1).

All mutant receptors, with the obvious exception of the S105A receptor, transiently expressed in COS-7 cells (about 5 x10^4 sites/cell) were able to stimulate phospholipase C under the action of Sar^1-AII, with K_{act} values quite similar to those obtained for the WT receptor (data not shown).

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Ligand		Kd (nM) WT (a)	S105A (a)	WT (b)	N111A (b)
Peptide agonist	[^{125}I] Sar^1-AII	0.76 ± 0.10	> 1000	0.55 ± 0.06	1.1 ± 0.2
Nonpeptide antagonists	[^{3}H] DUP 753	1.8 ± 0.2	nd	2.0 ± 0.8	> 200
	[^{3}H] LF 7-0156	3.0 ± 0.2	117 ± 7	nd	> 200
	[^{3}H] LF 8-0129	1.3 ± 0.2	11.7 ± 1.8	1.1 ± 0.2	> 200

nd = not determined.
a = intact CHO cells.
b = membranes from COS-7 cells.

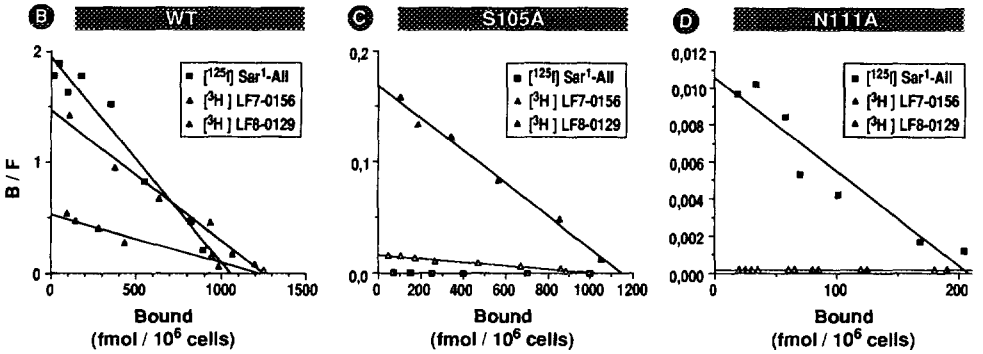

Figure 1 . Dissociation constants of peptide and nonpeptide radioligands for wild type and mutant AT_{1A} receptors.

Equilibrium binding experiments were carried out on intact CHO cells expressing the wild type (WT) or S105A mutant receptors or COS-7 cells transiently expressing the wild type (WT) or N111A mutant receptors, as described in Materials and Methods; the determinations represent the mean of triplicate assays. Each experiment was reproduced twice with similar results. Bmax values were about 1200 fmol/10^6 cells for intact CHO cells and 200 fmol/mg for COS-7 plasma membranes.

The K_d values for the various radioligands are collected in panel A. No significant differences were observed for WT receptors expressed either in CHO (a) or COS-7 cells (b). The most striking results demonstrating the differential effects of S105A and N111A mutations on peptide and nonpeptide recognition are illustrated by Scatchard plots in panels B : WT receptor in CHO cells ; C : S105A mutant receptor in CHO cells and D : N111A mutant receptor in COS-7 cells.

DISCUSSION

The present work demonstrates that the two amino acids Ser^{105} and Asn^{111}, located in the third transmembrane domain of the rat AT_{1A} angiotensin II receptor are critical for the differential recognition of angiotensin II and nonpeptide antagonists. Alanine replacement of Ser^{105} completely suppressed the ability of the receptor to bind angiotensin II, so that even the selection of stable transfectant clones was impossible, using the highest [^{125}I] Sar^1-AII concentrations; in contrast the S105A mutant receptor displayed much more preserved recognition of nonpeptide antagonists ; for instance, the increase in the K_d value for the high affinity ligand [^{3}H] LF 8-0129 did not exceed one order of magnitude.

A reverse pharmacological profile was shown for the N111A mutant, which possessed drastically reduced affinities for the radiolabeled nonpeptide antagonists [^{3}H] DuP 753 and the

original compounds from Fournier laboratories [^{3}H] LF 7-0156 (26) and [^{3}H] LF 8-0129. On the contrary, Ala replacement of Asn^{111} had no effect on receptor affinity for agonist or antagonist peptide ligands.

In agreement with the results of Ji et al. for the rat AT_{1B} receptor (20), we also found that Ser^{107} appears to play no role in peptide and nonpeptide ligand recognition. The S109A mutant receptor also possessed unchanged binding properties for all tested ligands.

None of the mutations of Ser^{107}, Ser^{109} and Asn^{111} into alanine had any significant effect on AII-induced inositol phosphate production.

The lack of incidence of the N111A mutation on AII recognition and signal transduction, together with the fact that perturbation of nonpeptide antagonist recognition has been assessed through direct radioligand binding, favour the hypothesis that Asn^{111} might directly interact with nonpeptide antagonists; this result may appear rather surprising if one considers that this amino acid is conserved among all AII receptors, including the AT_2 receptor and the Xenopus receptor (36) which have a poor affinity for nonpeptide AT_1-specific antagonists (37,38).

The results obtained with the mutant receptor S105A represent the first evidence for the role of a transmembrane domain amino acid in peptide binding. Here again, the questions arises whether Ser^{105}, which is conserved in AT_1 and Xenopus receptors but not in AT_2 receptors (36), directly interacts with peptide ligands or contributes, through intramolecular bonds, to an overall stabilization of the receptor structure.

Nevertheless, these differential effects of S105A and N111A mutations on peptide and nonpeptide binding confirm that different receptors epitopes or amino acids are involved in the recognition of the two classes of ligands (20, 21). Similar general conclusions were drawn from the data of two groups who performed a detailed analysis of chimeric or point-mutated receptors on the basis of sequence comparisons between mammalian or Xenopus receptors (36-38). Rather surprisingly, replacement of Val^{108} by Ile, its Xenopus counterpart, induced a much more pronounced reduction in receptor affinity for DuP 753 than Ala replacement of Ser^{107} which is absent in the Xenopus receptor (20). Schambye et al. (21) also differentiated the binding sites for angiotensin II and nonpeptide antagonists ; they demonstrated that the seventh transmembrane domain is essential for the binding of competitive nonpeptide antagonists, including DuP 753, and evidenced a prominent role of Asn^{295} in this process (21).

More generally, an increasing number of data point out specific amino-acids or domains differently involved in the recognition of peptide and nonpeptide ligands by G protein coupled receptors ; it holds true for neurokinin (8-15), opiate (18-19) and CCK (16,17) receptors.

The binding of both peptide and nonpeptide ligands to the AT_1 was significantly affected by the mutation of Lys^{102} into methionine. It remains to compare the role of Lys^{102} to that of Lys^{199} previously mentioned by Yamano et al. (39) as a candidate for interaction with the AII C-terminal carboxylate and whether this role is indirect or direct ; in the latter situation, it can be postulated that Lys^{102} might be involved in electrostatic interactions which would constitute initial events in multi-step processes underlying the binding of ligands possessing essential carboxylate groups.

All mutagenesis data, including those presented in the present paper, will be useful for the refinement of preliminary theoretical models (23). Concerted docking of peptide and

nonpeptide ligands should help to define the precise role of Ser^{105} in peptide agonist stimulation of the AT_1 receptor and Asn^{111} in the molecular events associated to the blockade of receptor activation by nonpeptide antagonists.

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