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Biochemical Pharmacology 65 (2003) 1329–1338
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Peptide and nonpeptide antagonist interaction with constitutively active human AT_1 receptors

Minh Tam Le[a,*], Patrick M.L. Vanderheyden[a], Márta Szaszák[b], László Hunyady[b], Veerle Kersemans[c], Georges Vauquelin[a]

[a]*Department of Molecular and Biochemical Pharmacology, Institute for Molecular Biology and Biotechnology, Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussel, Belgium*
[b]*Department of Physiology, Faculty of Medicine, Semmelweis University, P.O. Box 259, H-1444 Budapest, Hungary*
[c]*Laboratory of Radiopharmacy, University of Ghent, Ghent, Belgium*

Received 25 November 2002; accepted 22 January 2003

Abstract

Wild type human AT_1 receptors (WT-AT_1) and mutant receptors, in which Asn^{111} was replaced by glycine (N111G), alanine (N111A) and serine (N111S), or in which Asp^{281} was replaced by alanine (D281A) or in which N111G and D281A replacements were combined, were transiently expressed in CHO-K1 cells. While the biphenyltetrazole compound candesartan dissociated slowly and behaved as an insurmountable antagonist for WT-AT_1, it dissociated swiftly and only produced a rightward shift of the angiotensin Ang II- and -IV dose–response curves for inositol phosphate (IP) accumulation in cells expressing N111G. [^{3}H]candesartan competition binding yielded the same potency order of the related biphenyltetrazoles for WT-AT_1 and mutated receptors, i.e. candesartan > EXP3174 > irbesartan > losartan. Affinities were equal for WT-AT_1 and D281A and 40- to 400-fold lower for all Asn^{111} mutants. Mutations did not affect the affinity of the peptide antagonist [Sar^1Ile^8]Ang II (SARILE). Basal IP accumulation in cells with WT-AT_1 was not affected by any biphenyltetrazole antagonists and was increased by SARILE to 19% of the maximal Ang II stimulation. Basal IP accumulation was higher for cells expressing the Asn^{111}-mutated receptors. For N111G, this accumulation was partially inhibited by all the biphenyltetrazoles upon long-term (18 hr) exposure. In these cells SARILE produced the same maximal stimulation as Ang II. Asn^{111}-mutated AT_1 receptors are thought to mimic the pre-activated state of the wild type receptor and comparing the efficacy and affinity of ligands for such mutated receptors facilitate the distinction of partial (SARILE) and inverse (biphenyltetrazoles) agonists from true antagonists.

Keywords: CHO cells; AT_1 receptor; Nonpeptide antagonist; Surmountable; Insurmountable; Constitutive mutant

1. Introduction

Ang II, the main active peptide hormone of the renin–angiotensin system, exerts most of its biological functions by activating angiotensin II type 1 (AT_1) receptors. They contain seven α-helical transmembrane regions and, when stimulated by Ang II, this receptor will initiate a cascade of intracellular events starting with the activation of G_q, the increased production of IP and the release of calcium from intracellular stores. In the vasculature, this will result in a contractile response of the smooth muscle cells and lead to an increase in blood pressure [1]. Ang II is an important etiological factor in hypertension and other cardiovascular disorders, and therapeutic agents that are able to prevent the formation of Ang II or inhibit its interaction with the AT_1 receptor have proven to be highly successful to counter these pathologies. To this end, a fair number of nonpeptide AT_1 receptor antagonists have been developed during the 1990s. Among them, antagonists of the biphenyltetrazole class display considerable selectivity and high affinity for the AT_1 receptor but they appear to differ in their ability to reduce the maximal contractile response of vascular tissue in organ bath experiments [2–4]. Whereas antagonists like losartan only produce a rightward shift of

* Corresponding author. Tel.: +32-2-6291958; fax: +32-2-6291946.
E-mail address: tmleminh@vub.ac.be (M.T. Le).

Abbreviations: WT-AT_1, wild type human angiotensin II type 1 receptor; GPCR, G protein-coupled receptor; Ang II, angiotensin II (DRVYIHPF); SARILE, [Sar^1Ile^8]Ang II; TM, transmembrane helix; IP, inositol phosphate; DMEM, Dulbecco's modified Eagle's medium; ND, not determined; R, inactive receptor conformation; R′, pre-activated receptor conformation; R*, activated receptor conformation.

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doi:10.1016/S0006-2952(03)00072-8

the Ang II concentration–response curve (i.e. surmountable inhibition) others produce a partial to almost complete reduction in the maximal response (i.e. insurmountable inhibition).

Compared to contraction studies, cell lines expressing the endogenous or transfected AT_1 receptors offer major advantages for the investigation of its antagonists. In particular, their pharmacological properties can be investigated both by radioligand binding and by functional experiments under the same experimental conditions. To this end, Chinese Hamster Ovary cells expressing the human AT_1 receptor (CHO-AT_1 cells) have been used to compare the interaction of this receptor with the nonpeptide antagonists losartan, irbesartan, EXP3174 (the active metabolite of losartan) and candesartan [5]. It was found that all these antagonists were competitive inhibitors when co-incubated with Ang II and that all except losartan acted insurmountably when added to the cells ahead of Ang II. This latter behaviour could be ascribed to the slow reversibility of the antagonist binding [5,6]. To deal with the fact that the maximal response to Ang II could only be partially reduced by some antagonists, a two-state, two-step model was proposed in which the initial binding of all antagonists to the receptor is fast and reversible [7]. For insurmountable binding, this initial antagonist–receptor complex (L.R, which accounts for the surmountable inhibition) must further be converted into a tight binding state ($L.R_I$, which accounts for the insurmountable inhibition). While surmountable antagonists like losartan are unable to form tight binding complexes with the receptor, both states are in equilibrium for insurmountable antagonists. The $L.R_I$/L.R ratio is different for each of these antagonists and related to the stability of the tight binding complex. This model provides an adequate description of radioligand binding and functional experiments with CHO-K1 cells expressing the human AT_1 receptor [7].

Transfected cell systems also make it possible to perform mutation studies to explore the role of specific amino acids of the AT_1 receptor in its binding and functional properties. It was found that binding of Ang II is dependent on a series of residues located in the exterior part of the receptor and recent models suggest that the activation of the AT_1 receptor may proceed in two steps. A pre-activation step, which is characterised by the disruption of a constraining intramolecular interaction between Asn^{111} and TM VII, is proposed to allow the receptor to adopt a more relaxed conformation. It appeared that an interaction of Arg^2 of Ang II with Asp^{281} at the top of TM VII plays a crucial role in this transition. Subsequently full receptor activation is induced by the C-terminal part of Ang II [8]. In this respect, Lys^{199} has been shown to provide an essential contribution by forming a salt bridge with the terminal carboxyl group of Ang II in conjunction with a hydrogen bridge between the Phe^8 side chain and the His^{256} in TM VI [9]. In line with the syntopic interaction between nonpeptide antagonists and Ang II, Lys^{199} has also been found to play an important role in the binding and stabilisation of tight insurmountable nonpeptide antagonist–receptor complexes [10]. The aim of the present study is to explore whether the amino acids which are involved in the formation (Asp^{281}) and mimicking (Asn^{111}) of a pre-activated receptor conformation are also implicated in the differential binding properties of insurmountable and surmountable AT_1 receptor antagonists and of the peptide antagonist SARILE.

2. Materials and methods

2.1. Materials

2-Ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]-1*H*-benzimidazoline-7-carboxylic acid (candesartan), 2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole-5-carboxylic acid (EXP3174), 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-2-imidazolin-5-one (irbesartan), 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)-biphenyl-4-yl)methyl] imidazole (losartan) and [^{3}H]candesartan (17 Ci mmol^{-1}) were obtained from AstraZeneca. Ang II, Ang III, Ang IV and Ang-(1–7) were obtained from Neosystem. LipofectAMINE was from Invitrogen. Tyr^4 of SARILE was iodinated using the Iodogen® iodination reagent from Pierce and ^{125}I from ICN. Monoiodinated SARILE was isolated on a Hypersil BDS C18 reverse-phase HPLC column and stored at −20° in 10 mM KH_2PO_4, pH 6.5 containing 45% ethanol. All other chemicals were of the highest grade commercially available.

2.2. Mutagenesis of human AT_1 receptor DNA, cell culture and transient transfection

The human AT_1 receptor gene, cloned in the mammalian expression vector pcDNA3 (Invitrogen) was used for expression and mutagenesis. The mutations of human AT_1 DNA were created using the Mutagene kit (Bio-Rad). The DNA sequence of mutated receptor was confirmed by dideoxynucleotide sequencing (Amersham, Biosciences). Expression plasmids containing wild type and mutated AT_1 receptors were transiently transfected into CHO-K1 cells. The cells were cultured in DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 1% a solution of non-essential amino acids and 1 mM sodium pyruvate. The cells were grown in 5% CO_2 at 37° until 80% confluence on 12- or 24-well plates. Transfection was performed by replacing the culture medium with Opti-MEM I containing 8 μL mL^{-1} of LipofectAMINE and 1.5 μg mL^{-1} of purified plasmid DNA and then incubating for 5 hr. The supplemented DMEM was then added into transfected cells and the assays were carried out 2 days after transfection.

Table 1
Saturation and kinetic parameters of [^{3}H]candesartan binding to CHO-K1 cells transfected with the wild type and the mutant receptors

Receptors	K_d (nM) (saturation curves)	k_{obs} (M^{-1} min^{-1})	k_{-1} (min^{-1})	K_d (nM) (kinetic curves)
Wild type	0.27 ± 0.09	0.109 ± 0.009	0.006 ± 0.001	0.087 ± 0.021
N111G	4.41 ± 0.81	0.151 ± 0.025	0.090 ± 0.01	7.380 ± 2.04
N111A	3.69 ± 0.76	ND	ND	ND
N111S	2.48 ± 0.29	ND	ND	ND
N111G/D281A	3.89 ± 0.38	ND	ND	ND
D281A	0.32 ± 0.08	0.112 ± 0.028	0.007 ± 0.002	0.102 ± 0.047

2.3. Measurement of inositol phosphate accumulation

The transfected cells were labelled with 1 μCi mL^{-1} myo-[^{3}H]inositol (Amersham, Biosciences) 20 hr prior to the assay. After labelling, the cells were washed twice with DMEM and incubated with this medium containing 10 mM LiCl for 15 min at 37°. Then agonists were added and the cells were further incubated for 15 min. To investigate the effect of antagonists, the cells were pre-incubated with the antagonists for 30 min before starting incubation with various concentrations of the agonists. At the end of the incubation, the medium was removed, and total soluble IP was extracted from the cells as described previously [5]. The amount of [^{3}H]IP eluted from the columns was measured in a liquid scintillation counter.

2.4. Radioligand binding assays

Competition binding experiments were carried out on intact adherent cells as described previously [5]. Briefly, the cells were incubated at 37° for 60 min with 1.5 nM [^{3}H]candesartan (for the wild type and D281A mutant) or 5 nM [^{3}H]candesartan (for N111G, N111A, N111S and N111G/D281A mutants) in the presence of unlabelled ligands. Saturation binding was performed with [^{3}H]candesartan at concentrations ranging between 0.15 and 15 nM. For the wild type and the D281A mutant, kinetic (association and dissociation) experiments were performed using 1.5 nM [^{3}H]candesartan. Dissociation was measured after addition of 1 μM unlabelled candesartan. Binding of 0.5 nM [^{125}I]SARILE to the wild type and N111G receptors was performed in Hepes-DMEM containing 0.1% BSA at 37° for the indicated periods of time. Non-specific binding was measured in the presence of 1 μM unlabelled candesartan. At the end of incubation, the cells were washed three times with Krebs–Ringer buffer at 4°, solubilised with 0.2 M sodium hydroxide (500 μL/well), and the cell-bound radioactivity was measured for 3 min in a liquid scintillation counter.

2.5. Data analysis

The binding and functional parameters were calculated by non-linear regression analysis using Graph Pad Prism. Data points were the mean ± SEM of three to eight separate experiments with duplicate or triplicate determinations. The sum of the relative variation of the association (k_{obs}) and dissociation (k_{-1}) rate constants are used to calculate the variation of the corresponding K_d values in Table 1. These K_d values are calculated according to the equation: $K_d = k_{-1}[L]/(k_{obs} - k_{-1})$.

3. Results

3.1. Binding experiments with [^{3}H]candesartan

Wild type human AT_1 and mutant receptors, in which Asn^{111} was replaced by glycine (N111G), alanine (N111A) and serine (N111S), or in which Asp^{281} was replaced by alanine (D281A) or in which N111G and D281A replacements were combined, were transiently expressed in CHO-K1 cells. Saturation binding experiments with the potent nonpeptide AT_1 receptor antagonist [^{3}H]candesartan revealed a single class of binding sites for all receptor constructs. The calculated K_d values were in the sub-nanomolar range for the wild type and D281A and in the nanomolar range for all mutants with substituted Asn^{111} (Table 1). Because of the long delay for equilibrium of [^{3}H]candesartan saturation binding (particularly of the low radioligand concentrations), the corresponding K_d values were only apparent [11]. Therefore the K_d values for the wild type and D281A receptors were calculated as based on kinetic experiments. The pseudo-first-order rate constants for association (k_{obs}) and the dissociation rate (k_{-1}) allowed to calculate the kinetic K_d values of [^{3}H]candesartan. For the wild type receptor and for D281A, these K_d values were similar and below those obtained from saturation binding experiments. Similar kinetic experiments were carried out for N111G. Compared to the wild type receptor, the dissociation was appreciably faster for N111G and the K_d value of [^{3}H]candesartan for this mutant was comparable when calculated from the kinetic data or obtained from saturation binding experiments (Table 1).

All competition binding experiments with [^{3}H]candesartan yielded monophasic curves. The unlabelled nonpeptide antagonists showed a similar potency order for wild type and mutated AT_1 receptors, i.e. candesartan > EXP3174 > irbesartan > losartan. As shown in Table 2, all of the nonpeptide antagonists bound with high affinity to the wild type receptor with K_i values ranging from 0.09 to 4.47 nM. The D281A mutation only minimally affected

Table 2
Binding affinities of agonists and antagonists for the human wild type and the mutated AT_1 receptors

Ligands	K_i (nM, mean ± SEM, N = 3–8)					
	WT AT_1-R	N111G	N111A	N111S	N111G/D281A	D281A
Peptide agonist						
Ang II	12.3 ± 1.3	0.85 ± 0.25	1.10 ± 0.38	2.12 ± 0.37	1.75 ± 0.77	>1000
Ang III	24.5 ± 8.0	4.33 ± 1.29	4.14 ± 1.82	3.78 ± 1.08	4.90 ± 1.97	>1000
Ang-(1–7)	1796 ± 260	2310 ± 458	>1000	>1000	>1000	>1000
Peptide antagonist						
SARILE	0.59 ± 0.13	0.19 ± 0.01	1.02 ± 0.37	0.84 ± 0.37	9.8 ± 2.0	65.4 ± 9.8
Non-peptide antagonist						
Candesartan	0.09 ± 0.01	3.37 ± 0.71	5.21 ± 0.33	3.61 ± 0.81	5.85 ± 1.15	0.18 ± 0.09
EXP3174	0.18 ± 0.01	9.97 ± 1.13	67.5 ± 7.7	34.3 ± 6.1	45.9 ± 4.4	0.33 ± 0.11
Irbesartan	0.44 ± 0.07	17.2 ± 3.0	123 ± 26	50.3 ± 8.8	63.5 ± 23.5	0.83 ± 0.32
Losartan	4.47 ± 0.46	212 ± 9	533 ± 127	518 ± 140	548 ± 130	4.57 ± 1.31

K_i values were calculated from the IC_{50} of [^{3}H]candesartan competition curves with corresponding K_d values according to Cheng and Prusoff [32].

the K_i values for the different antagonists. On the other hand, all the mutants with substituted Asn^{111} displayed a 40- to 400-fold decreased affinity for all nonpeptide antagonists. Fig. 1 compares the increase of the K_i values of losartan and candesartan by the latter mutations.

K_i values of Ang II were more than six times lower for all mutants with substituted Asn^{111} as compared to the wild type. The K_i value of Ang II was markedly increased for the D281A mutant but this effect was completely alleviated by an additional N111G substitution (Table 2). The Ang II analog SARILE displayed very similar K_i values for wild type and the N111G, N111A, N111S mutants. Its K_i value was 110-fold higher for D281A and this effect was only partially attenuated by the introduction of an additional N111G substitution (Table 2). Ang III and the C-terminal truncated Ang II fragment, Ang-(1–7), displayed the same, low affinity for the wild type and the N111G mutant (Table 2).

3.2. Binding experiments with [^{125}I]SARILE

Despite the similar K_i values of SARILE for the wild type and N111G, kinetic experiments with [^{125}I]SARILE revealed that association and dissociation of this radioligand was much faster for the wild type receptor (Fig. 2).

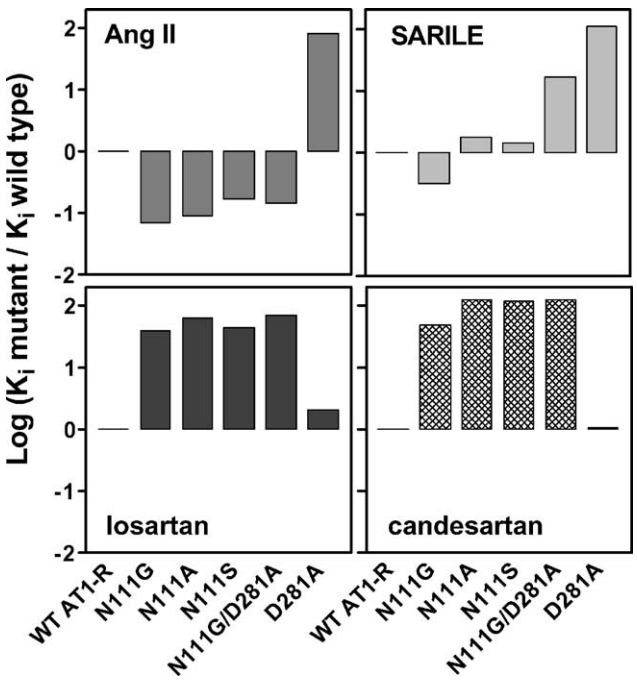

Fig. 1. Competition binding of [^{3}H]candesartan to the wild type and different mutants performed at 37° for 60 min in the presence of Ang II, SARILE, losartan and candesartan. The K_i values are expressed as log(K_i mutant/K_i wild type).

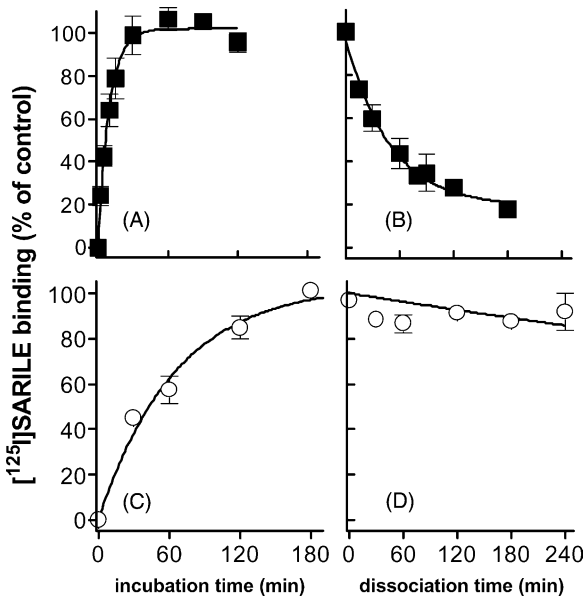

Fig. 2. Association rate (A, C) and dissociation rate (B, D) experiments of [^{125}I]SARILE binding on the wild type (■) and N111G mutant (○). Cells were incubated with 0.5 nM [^{125}I]SARILE in Hepes-DMEM medium containing 0.1% BSA at 37° for different period of time for association experiments. Dissociation of [^{125}I]SARILE from the cells was started by the addition of 1 μM unlabelled candesartan. Values are given as percentages of the specific binding obtained by subtracting the total binding from the non-specific binding.

Table 3
Basal and maximal stimulation of IP production by angiotensin

Receptors	Basal	Ang II	Ang III	Ang IV
CHO-K1	203 ± 21	202 ± 18	ND	ND
Wild type	201 ± 21	957 ± 88	902 ± 57	961 ± 66
N111G	387 ± 24	798 ± 45	702 ± 48	762 ± 106
N111A	282 ± 13	693 ± 32	609 ± 48	673 ± 48
N111S	330 ± 23	737 ± 60	640 ± 60	815 ± 63
N111G/D281A	382 ± 21	699 ± 47	718 ± 32	641 ± 119
D281A	209 ± 25	666 ± 67	539 ± 15	655 ± 111

The values represent total inositol phosphates (in cpm) measured in CHO-K1 cells transfected with vector alone, with wild type or mutated AT_1 receptors. The cells were incubated at 37° for 15 min in the absence (basal accumulation) or presence of 10 μM Ang II, Ang III or Ang IV.

The corresponding k_{obs} and k_{-1} values for the wild type were $0.14 \pm 0.01\ M^{-1}\ min^{-1}$ and $0.021 \pm 0.006\ min^{-1}$, respectively. The derived K_d value (0.17 ± 0.05 nM) was close to the K_i value obtained from SARILE/[^{3}H]candesartan competition experiments. The dissociation of [^{125}I]SARILE from N111G was too slow to calculate the kinetic parameters and the K_d value of this radioligand.

3.3. Functional study

Receptor activation was monitored by measuring the accumulation of IP in intact adherent cells (15 min incubation at 37°). The basal accumulation was the same in untransfected and mock transfected CHO-K1 cells and in cells expressing the wild type and D281A but it was elevated in cells expressing the mutants with a substituted Asn^{111} (Table 3). Maximal Ang II, Ang III and Ang IV stimulated IP accumulation was similar for all receptors (Table 3). Concentration–response curves yielded similar EC_{50} values for Ang II and Ang III for wild type and Asn^{111}-mutated receptors (Table 4). Whereas Ang IV was a weak agonist for the wild type, it was a potent agonist for the different Asn^{111}-mutated receptors with EC_{50} values that ranged between 2.6 and 6 nM.

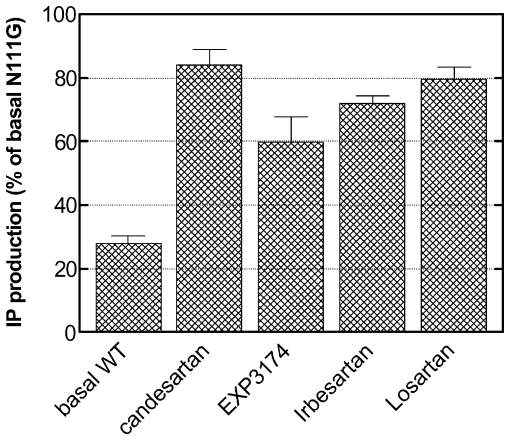

Fig. 3. Inverse agonist properties of nonpeptide antagonists on the N111G mutant receptor. Ten micromolar of the nonpeptide antagonists and 1.5 μCi mL^{-1} myo-[^{3}H]inositol were added to the cells during 18 hr at 37° prior to the measurement of the IP accumulation. The values of basal IP accumulation are expressed as percentages of control (basal accumulation of N111G in the absence of the antagonist) from four independent experiments. Basal IP accumulation of transfected wild type AT_1 receptor is also represented. All values were significantly different from the control as determined by a nonparametric *t*-test with two-tailed *P*-values ($P \leq 0.05$).

At concentrations up to 10 μM, the nonpeptide AT_1 receptor antagonists candesartan, EXP3174, irbesartan and losartan did not affect basal IP accumulation in cells expressing the wild type or the N111G mutant when the incubation time was 15–60 and 120 min (data not shown). In similar experiments, a significant decrease in IP accumulation for N111G mutant was observed upon 18 hr incubation with these antagonists (Fig. 3). In the experiment shown in Fig. 4, cells were pre-incubated for 30 min with a fixed concentration of candesartan and subsequently incubated for 15 min with the antagonist in the presence of

Table 4
EC_{50} values of Ang II, Ang III, and Ang IV-mediated IP accumulation in CHO-K1 cells expressing the wild type, N111G, N111A, N111S, N111G/D281A or D281A mutated human AT_1 receptors

Ligand	Wild type	N111G	N111A	N111S	N111G/D281A	D281A
EC_{50} (nM)						
Ang II	1.25 ± 0.18	1.23 ± 0.4	1.66 ± 0.49	1.46 ± 0.24	2.12 ± 0.49	50 ± 19
+0.1 μM candesartan		44.3 ± 9.6	16.0 ± 2.7	50.5 ± 15.1	51.2 ± 18.1	ND
+1 μM candesartan		274 ± 80	139 ± 38	316 ± 74	568 ± 185	ND
Ang III	2.76 ± 0.59	1.70 ± 0.13	1.07 ± 0.2	0.63 ± 0.13	2.78 ± 0.72	247 ± 20
Ang IV	1125 ± 204	2.59 ± 1.59	3.81 ± 0.66	6.01 ± 1.18	1.29 ± 0.44	3354 ± 882
+0.1 μM candesartan		21.5 ± 4.7	70 ± 33	117 ± 5	65 ± 30	ND
+1 μM candesartan		110 ± 20	589 ± 74	929 ± 273	409 ± 211	ND
Inhibition parameters of candesartan						
Ang II/pA_2		8.94	7.96	8.72	8.36	
Slope		0.78 ± 0.18	0.96 ± 0.16	0.85 ± 0.07	0.96 ± 0.33	
Ang IV/pA_2		8.07	8.18	8.32	8.68	
Slope		0.78 ± 0.15	1.01 ± 0.19	0.87 ± 0.14	0.89 ± 0.38	

The cells were stimulated at 37° for 15 min with the peptides alone or pre-incubated for 30 min with candesartan before the stimulation.

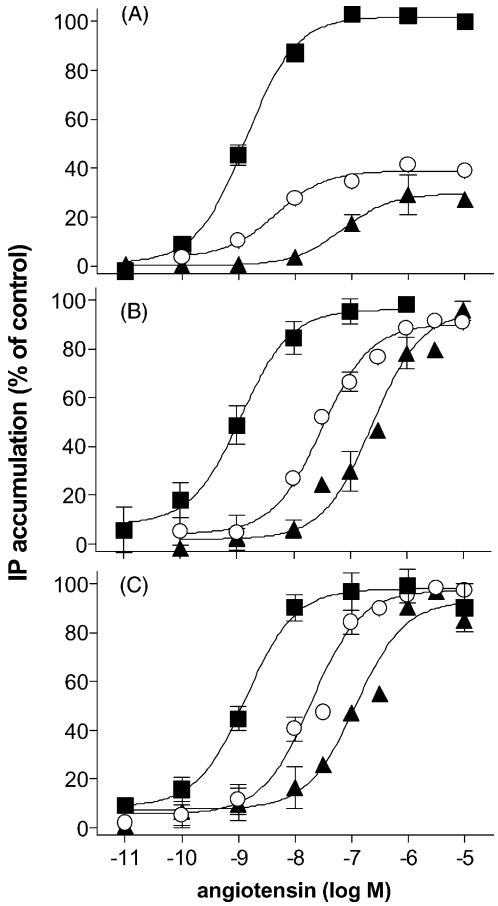

Fig. 4. Effect of candesartan on Ang II and Ang IV concentration–response curves. (A) CHO cells transfected with the wild type AT_1 receptor were pre-incubated for 30 min at 37° either alone with medium (■) or with 1 nM (○) or 10 nM candesartan (▲). The cells were then further challenged with increasing concentrations of Ang II for 15 min. (B) Similar Ang II concentration–response curves on N111G mutant after pre-incubation of the cells with medium (■), 0.1 μM candesartan (○) or 1 μM candesartan (▲) for 30 min. (C) Ang IV concentration–response curves on the N111G mutant after pre-incubation of the cells with medium (■), 0.1 μM candesartan (○) or 1 μM candesartan (▲) for 30 min. Data are expressed in percent of control (i.e. maximal stimulation by the agonists).

increasing concentrations of Ang II or Ang IV. Under these conditions, 1 and 10 nM candesartan reduced the maximal response of the wild type to Ang II by 61 and 73%, respectively (Fig. 4A). In agreement, inhibition experiments in which cells expressing the wild type were pre-incubated with increasing concentrations of candesartan and then incubated with the antagonist and a fixed concentration of Ang II yielded a biphasic curve (Fig. 5). The most potent component of this curve has been shown to reflect insurmountable inhibition [6] and affected 75% of the response in this case. No such experiments could be performed with Ang IV because of its low potency for the wild type receptor. In contrast, candesartan only produced parallel rightward shifts of the concentration–response curves of Ang II and Ang IV for the N111G mutant (Fig. 4B and C, respectively) as well as for all other Asn^{111} mutants. Table 4 summarises the effect of 0.1

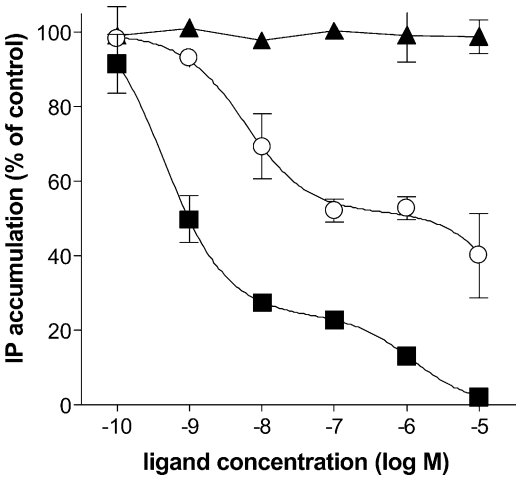

Fig. 5. Antagonist concentration-inhibition curves of Ang II on the wild type expressing CHO-K1. The cells were pre-incubated with increasing concentrations of candesartan (■), SARILE (○) or Ang-(1–7) (▲) for 30 min at 37°, and then 10 μM Ang II was added. The incubation was continued for 15 min and the inositol phosphate production was measured.

and 1 μM candesartan on the agonist EC_{50} values, and the corresponding pA_2-values calculated by Schild regression analysis for the Asn^{111} mutants. These pA_2-values are very similar as those obtained by saturation binding (Table 1) and/or by competition binding experiments (Table 2).

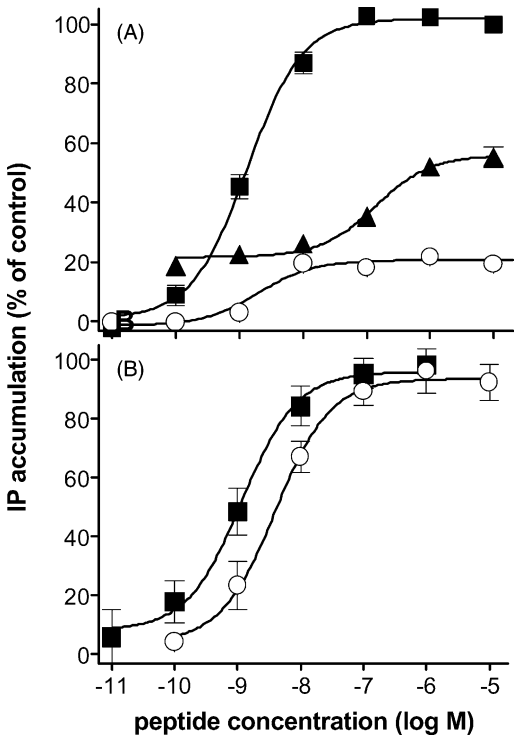

Fig. 6. Concentration–response curves of SARILE in the wild type (A) and the N111G mutant (B). The cells were incubated with increasing concentrations of Ang II (■) or SARILE (○) at 37° for 15 min. The ability of SARILE to block the Ang II response in the wild type cells was performed by pre-incubation of the cells with 0.1 μM SARILE (▲ in panel A only).

SARILE produced a concentration-dependent and saturable increase in IP production in cells expressing the wild type AT_1 receptor but the maximal response was only 19% of that of Ang II (Fig. 6A). Because of its low efficacy, SARILE was also able to inhibit the Ang II responses. As for candesartan, pre-incubation of the cells with SARILE decreased the maximal effect of Ang II and this insurmountable inhibition was partial as it only affected 45% of the maximal response (Fig. 6A). Similar to the wild type, SARILE possessed minimal agonist activity for D281A (data not shown). In contrast, SARILE acted as a full agonist for the N111G with an EC_{50} value of 5.21 ± 1.57 nM (Fig. 6B). Ang-(1–7) did not induce a measurable IP accumulation in wild type or N111G mutant (data not shown) and did not affect Ang II responses at concentrations up to 10 μM (Fig. 5).

4. Discussion

Both agonist- and antagonist-AT_1 receptor interactions are currently thought to represent multistep processes and can conveniently be described by two-state models. In the present study we show that replacement of the wild type receptor by constitutively activated mutants that mimic its pre-activated state has the same effect on surmountable and insurmountable nonpeptide antagonists. On the other hand, the effect of these mutations on SARILE, which behaves primarily as a peptide AT_1 receptor antagonist but still possess its own weak agonist activity, are much closer to those observed for the full agonist Ang II.

A two-step model for AT_1 receptor activation was first proposed by Noda *et al.* [12] and further refined by Le *et al.* [8]. It is now assumed that the basal state of the receptor (R) is stabilised by a constraining interhelical interaction between Asn^{111} in TM III and Tyr^{292} or Asn^{295} in TM VII [13,14]. In the initial process of receptor activation, this interaction is disrupted by the interaction between Arg^2 of Ang II and Asp^{281} located in TM VII of the receptor. The so-formed pre-activated state of the receptor (R′) is considered to adopt a more "relaxed" conformation and to provide a more appropriate binding pocket for the C-terminal residues of Ang II. Recent experiments with Ang II fragments like Ang III and Ang IV have stressed the importance of these residues in switching R′ into the fully active conformation (R^*). In this respect, Asn^{111} is considered to play a dual role; it stabilises R and, once liberated (in R′), it contributes as an agonist switch to the formation and/or stabilisation of R^* by interacting with Tyr^4 of Ang II [12]. In addition, evidence has been presented for the terminal carboxyl group of Ang II to form a salt bridge with Lys^{199} in TM V of the receptor. This allows an appropriate positioning of the aromatic side chain of Phe^8 to undergo hydrogen bonding with His^{256} in TM VI, the second agonist switch, which is required for the full activation process [9,15].

The pre-activated state of the AT_1 receptor, in which the constraining intramolecular interaction between Asn^{111} and TM VII is disrupted, can be mimicked by substituting Asn^{111} by amino acids such as glycine (N111G), alanine (N111A) and serine (N111S). These receptor mutants are constitutively active, as reflected by the increased IP production under basal conditions in this and other studies [12–14]. This phenomenon is not restricted to AT_1 receptors as substitution of equivalent residues to Asn^{111} in rhodopsin (Glu^{113}), α_{1B}-adrenergic receptors (Asp^{113}), bradykinin B2 (Asn^{113}), CXCR4 (Asn^{119}) and platelet-activating factor (Asn^{100}) receptor also causes their constitutive activation [16–21]. Asn^{111}-mutated AT_1 receptors appear to bear the same structural features of the pre-activated wild type receptor. In this respect, the idea that receptor pre-activation provides a better alignment of its binding pocket for the C-terminal moiety of Ang II is based on the increased affinity and potency of this agonist and its C-terminal fragments Ang III, Ang IV and Ang-(4–8) to produce full activation of the receptor mutants. Similarly this mutation also increases the affinity for CGP42112A, a AT_2 selective peptide ligand which mimics the C-terminal part of Ang II [14,22,23]. When considering that the Asn^{111} mutants reflect the pre-activated state of the receptors these data indicate that the C-terminal side of Ang II plays an essential role subsequent to receptor pre-activation. This is consistent with the observation that the C-terminal truncated Ang II fragment, Ang-(1–7), displays the same low affinity for the wild type and the N111G mutant (Table 2) and that it cannot activate the receptor. On the other hand, because of the 45–200 times decreased affinity of losartan for the N111A mutant, the pre-activation process is considered to provoke a misalignment of amino acid residues involved in the binding of this nonpeptide antagonist [12–14,22,23]. In the present study, we found that the different Asn^{111} mutations (i.e. N111G, N111A and N111S) caused a similar decrease of the affinity for four nonpeptide antagonists of the biphenyltetrazole class. Moreover, the order of affinities of these antagonists for the wild type (i.e. candesartan > EXP3174 > irbesartan > losartan) remained preserved for the Asn^{111} mutants (Table 2). It is, therefore, likely that the binding of a common structural moiety of these antagonists—i.e. their biphenyltetrazole moiety—is preferentially affected by the structural change accompanied with the Asn^{111} mutation. The additional epitopes of these antagonists are structurally distinct and may be held responsible for their difference in affinity for the AT_1 receptors.

Biphenyltetrazole compounds have been designed with the intention to mimic certain structural features of Ang II and, in this respect, the rationale for introducing an acidic tetrazole function was based on its potential to mimic the carboxyl group in Asp^1 or the hydroxyl group in Tyr^4 of the agonist [3]. However, the fact that replacing Asn^{111} by glycine, alanine or serine has the same effect on the affinity

of each compound (Table 2), suggests that this amino acid is not directly involved in the binding of the tetrazole moiety and, in this respect, amino acids like Arg^{167} have already been proposed as alternative candidates [25,26]. Instead, the present data point towards a structural role of Asn^{111} with respect to nonpeptide antagonist binding. Their decreased binding affinity to the Asn^{111}-mutated receptors could be attributed to the disruption of the interaction between Asn^{111} and TM VII. In addition TM III may also play a more general structural role with respect to the modulation of the biphenyltetrazole antagonist binding, since the substitution of other amino acids surrounding Asn^{111} (i.e. Ser^{107}, Val^{108}, Ser^{109} and Ser^{115}) have also been reported to reduce the binding affinity of losartan [22–24]. Based on similar mutation studies, several additional amino acids belonging to TM IV, V, VI and VII have been found to affect the binding of losartan without altering the binding of Ang II [27,28]. Most of them are positioned within a small distance from each other within a plane that is one or two helical turns below the membrane surface.

Whereas losartan is only able to undergo loose, surmountable binding to the wild type AT_1 receptors, the other antagonists i.e. irbesartan, EXP3174 and candesartan have been found to trigger a tight binding state of this receptor [7]. Such slowly dissociating antagonist–receptor complexes have been held responsible for the partial (irbesartan and EXP3174) or almost complete (candesartan) insurmountable inhibition of the Ang II response. In this respect, kinetic experiments (Table 1) reveal that the decreased affinity for candesartan for the N111G mutant is associated with a substantial increase in its dissociation rate. In agreement with this observation, pre-incubation with candesartan no longer decreases the maximal Ang II response for this receptor mutant. Instead, it only causes a rightward shift of the Ang II concentration–response curves. Similar surmountable inhibition by candesartan is also observed for the concentration–response curves of Ang IV. Schild regression analysis yielded pA_2 values for candesartan between 8 and 9 (Table 4). This potency corresponds to the K_d value of [^{3}H]candesartan (Table 1) as well as to the K_i value of candesartan in competition binding experiments (Table 2). These results suggest that the nonpeptide antagonist-binding site of the Asn^{111}-mutated receptor at least partially overlaps with the site that accommodates the six C-terminal amino acids of Ang II. An overlap between the binding site for the nonpeptide antagonists and for Ang II has also been reported for the wild type receptor [10,29,30] and earlier mutation studies have already shed light on the important role of common residue of the receptor, Lys^{199}, in the binding of Ang II and the nonpeptide antagonists. This fits with the earlier evoked hypothesis that the carboxyl group of such antagonists mimics the terminal carboxyl group of Ang II [3] and with the interaction of the latter one with Lys^{199}. For Ang II and related agonists this interaction facilitates the optimal positioning of the side chain of Phe^8, which is a major requirement for receptor activation. On the other hand, for peptide and nonpeptide antagonists, this interaction will merely contribute to the energy of ligand binding.

Mutation of Asp^{281}, located at the extracellular end of the TM VII of the receptor was previously shown to interfere with agonist-mediated receptor activation [8,31]. In the present study, substitution of this amino acid by Ala does not affect the binding properties of [^{3}H]candesartan (Table 1) or any of the biphenyltetrazole antagonists (Table 2) but it produces a profound decrease in the affinity of Ang II and Ang III. This has been taken as evidence for a strong interaction between Asp^{281} and Arg^2 of Ang II, a process which allows the agonist to disrupt the R-state stabilising interaction between Asn^{111} and TM VII and, consequently, to drive the receptor in its pre-activated conformation R′ [8]. Yet, even Asp^{281}-mutated receptors can be forced to reside in the R′ state by introducing the additional Asn^{111} mutation. This explains why the N111G/D281A double mutant and single Asn^{111}-mutated AT_1 receptors display the same high potency for agonists like Ang II, Ang III and Ang IV and low potency for the nonpeptide antagonists (Table 2).

Taken together, the present findings all suggest that the nonpeptide AT_1 receptor antagonists preferentially bind to the inactive conformation (R) of the receptor than to its pre-activated conformation (R′) and, consequently, that they have the potential to act as inverse agonists. Since the transfection of CHO-K1 cells with the wild type AT_1 receptor did not result in a measurable elevation of basal IP accumulation, inverse agonism could not be assessed in this system. Because of their constitutive activity, Asn^{111}-mutated AT_1 receptors offer an alternative approach to assess the potential inverse agonistic properties of the biphenyltetrazole compounds. In this respect, it has already been reported that the basal IP accumulation is reduced by nanomolar concentrations of EXP3174 after 18 hr incubation in COS-1 cells transfected with the N111G mutated rat AT_{1A} receptor [12]. In the present study on CHO-K1 cells transfected with the N111G mutated human AT_1 receptor, all biphenyltetrazole compounds tested were also able to suppress the basal IP production after 18 hr and this irrespective of their classification as surmountable or insurmountable antagonists for wild type receptors. However, when the cells were only exposed for 2 hr to these antagonists, they produced no change in the basal IP production. The present findings lend support to the suggestion by Noda *et al.* [12] that the interconversion between R′ and R might be extremely slow in the N111G mutant. This is possibly related to the fact that R-stabilising intramolecular interactions are incapacitated in this mutant. However, in another study on COS-7 cells transfected with the N111A mutated rat AT_{1A} receptor [14], a 1 hr incubation was already sufficient for micromolar concentrations of losartan to produce a partial reduction of the basal IP accumulation. The reasons for this divergence are presently unknown but, in the first

place, they could be related to differences in experimental conditions including the use of different host cells.

The octapeptide SARILE was initially considered to behave as an antagonist and, in its radioiodinated form, it is widely used for the labelling of AT_1 receptors in radioligand binding studies. More recently it has been shown to produce modest activation of the wild type rat AT_1 receptor in cell lines [12]. In agreement, it also behaves as a weak partial agonist for the wild type human AT_1 receptor, with a maximal stimulatory effect of 19% of that of Ang II. Because of its low efficacy, SARILE is able to inhibit the Ang II-mediated IP accumulation in a concentration-dependent fashion. The concentration-inhibition curve of SARILE is biphasic with a plateau that exceeds its own maximal effect. In analogy with the nonpeptide antagonists, this suggests that the antagonistic effect of SARILE on the wild type is partially insurmountable and, hence, that it is capable of forming fast- and slow-dissociating complexes with the receptor. However, it remains to be established whether the SARILE bound receptor adopts the same states/conformations as with the nonpeptide antagonists. In this respect, experiments with mutated AT_1 receptors shed light on some marked differences between the binding properties of SARILE and the nonpeptide antagonists. First, whereas the nonpeptide antagonists have a clear preference for the inactive receptor, the binding affinity of SARILE remains virtually unchanged for all Asn^{111}-mutated receptors. A second difference is that the affinity of SARILE is strongly reduced by substitution of Asp^{281} and restored by a second mutation affecting Asn^{111}. This behaviour is similar to that of Ang II suggesting that an interaction between Arg^2 of SARILE and Asp^{281} is also an important step in its binding process. As in earlier studies on Asn^{111}-mutated AT_1 receptors [12], SARILE becomes a full agonist for the N111G mutant. Since this receptor mutant is thought to reflect the pre-activated form of the wild type receptor, it may be deduced that the side chain of Ile^8 is able to function as an effective agonist switch once the receptor has acquired the pre-activated form. To explain the weak partial agonistic activity of SARILE on the wild type receptor, it could therefore be questioned whether the interaction of its Arg^2 side chain with Asp^{281} is sufficiently ''productive'' to drive all the receptors in the pre-activated state. In this respect, it is of interest to notice that SARILE displays the same affinity for the wild type and Asn^{111}-mutated AT_1 receptors while the affinity of Ang II is appreciably higher for the mutated receptors. It is therefore plausible that the difference in affinity of the ligand for R and R′ will have an impact on the degree of receptor pre-activation and, consequently on its final activity.

In conclusion, while AT_1 receptor-pre-activating mutations of Asn^{111} increase its affinity of Ang II, they do not affect its affinity for SARILE and even decrease its affinity for all biphenyltetrazole compounds tested in this study. This fits with their respective functional properties as full agonist, partial agonist and inverse agonists for the wild type receptor. Moreover, SARILE gains the status of full agonist while the biphenyltetrazole compounds behave as inverse agonists for these receptor mutants. Comparing the efficacy and affinity of ligands for the wild type and Asn^{111}-mutated AT_1 receptors might represent sensitive and complementary approaches to distinguish partial and inverse agonists from true antagonists.

Acknowledgments

We are most obliged to AstraZeneca Sweden, the Queen Elizabeth Foundation Belgium, the Fund for Scientific Research Flanders, Belgium, the Research Council of the VUB and the Hungarian Ministry of Health. We thank S. Meloche and G. Guillemette for kindly providing the human AT_1 receptor gene. We thank F.L. Fierens for his contribution to this work.

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