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"Network Leaning" as a Mechanism of Insurmountable Antagonism of the Angiotensin II Type 1 Receptor by Non-peptide Antagonists*

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A mechanistic understanding of the insurmountable antagonism of the angiotensin II type 1 (AT_1) receptor could be fundamental in the quest for discovery and improvement of drugs. Candesartan and EXP3174 are competitive, reversible insurmountable antagonists of the AT_1 receptor. They contain di-acidic substitutions, whereas the surmountable antagonist, losartan, contains only one acidic group. We tested the hypothesis that these two classes of ligands interact with the AT_1 receptor through similar but not identical bonds and that the differences in the acid-base group contacts are critical for insurmountable antagonism. By pharmacological characterization of site-directed AT_1 receptor mutants expressed in COS1 cells we show that specific interactions with Gln^{257} in transmembrane 6 distinguishes insurmountable antagonists and that abolishing these interactions transforms insurmountable to surmountable antagonism. In the Q257A mutant, the dissociation rate of [^{3}H]candesartan is 2.8-fold more than the rate observed with wild type, and the association rate was reduced 4-fold lower than the wild type. The pattern of antagonism of angiotensin II concentration-response in the Q257A mutant pretreated with EXP3174 and candesartan is surmountable. We propose that leaning ability of insurmountable antagonists on Gln^{257} in the wild-type receptor is the basis of an antagonist-mediated conformational transition, which is responsible for both slow dissociation and inhibition of maximal IP response.

Antagonism of G-protein-coupled receptors (GPCRs)[1] such as the angiotensin II type 1 receptor (AT_1) has gained much attention, evidently due to therapeutic success of the drugs targeting this family of receptors. Antagonists of GPCRs exhibit diverse patterns of antagonism that range from the classical surmountable antagonism (parallel rightward shift of agonist concentration-response curves with no decline of the maximum response) to insurmountable antagonism (partial to complete waning of the response accompanying a seeming rightward shift of the agonist response curve). Insurmountable antagonist occupancy of the receptor generally prolongs the time taken for re-sensitization to agonist than the surmountable antagonist occupancy (1). Common mechanisms proposed for insurmountable antagonism involve phenomena attributed to equilibrium between allosteric activity states of receptors that (i) affect the kinetics of drug-receptor interaction, (ii) induce antagonist-dependent receptor conformation that is refractory to agonist binding, and (iii) modulate coupling to unknown molecules that stabilize the antagonist-bound state of the receptor (2–5). In other instances the insurmountable antagonists forming a covalent bond with the receptor (irreversible) or the insurmountable antagonists dissociating very slowly from the receptor (pseudo-irreversible) have been proposed (2). Even so, the molecular mechanism responsible for distinct efficacy from different types of drug-receptor complexes has remained unclear.

Pharmacological behavior of the non-peptide antagonists of the AT_1 receptor provides several advantages as a model to study insurmountable antagonism of GPCRs. AT_1 receptor antagonists displaying both types of pharmacological behavior are currently being evaluated for therapeutic potential in cardio-protective, reno-protective, anti-proliferative, and anti-fibrotic effects in addition to anti-hypertensive effects (2–5). Insurmountable antagonism is not unique to the AT_1 receptors. In fact it has been reported for GPCR systems that include those for noradrenaline, histamine, acetylcholine, 5-hydroxy tryptamine (5-HT), substance P, bradykinin, cholecystokinin, and chemotactic C5α receptors (2–5), suggesting that a mechanistic understanding of this important pharmacological phenomenon could be fundamental in the quest for discovery and improvement of drugs. Both classes of the AT_1 receptor antagonists share a structure with biphenyltetrazole linked to a planar substituted imidazole with a short alkyl chain (Fig. 1*A*). Candesartan and EXP3174 are competitive, reversible antagonists that display insurmountable antagonism (2–5). They contain di-acidic substitutions, whereas the surmountable antagonist losartan contains only one acidic group. In aortic contraction as well as inositol phosphate (IP) production assays, preincubation with losartan produces classical surmountable antagonism, whereas candesartan and EXP3174 produce insurmountable antagonism. Co-incubation experiments and detailed kinetic analysis have lead to several proposals for the mechanism mentioned earlier (2–5).

A difference in drug-receptor-bonding interactions due to differences in the acid-base groups is one possible mechanism. We speculate that the two classes of ligands interact with the AT_1 receptor through similar but not identical bonds, and the difference in contact is critical for their pharmacological properties. In a previous study (6) we demonstrated that the tetrazole moiety of losartan interacts primarily with Lys^{199} and secondarily with His^{256} (see Fig. 1). Candesartan contains an

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[1] The abbreviations used are: GPCR, G-protein-coupled receptor; Ang II, angiotensin II (DRVYIHPF); WT, wild type; AT_1, angiotensin II type 1; TM, transmembrane; EC, extracellular; IP, inositol phosphate; Rg, ground state inactive receptor conformation; R, basal partially active conformation; R*, active receptor conformation; Sar, sarcosine.

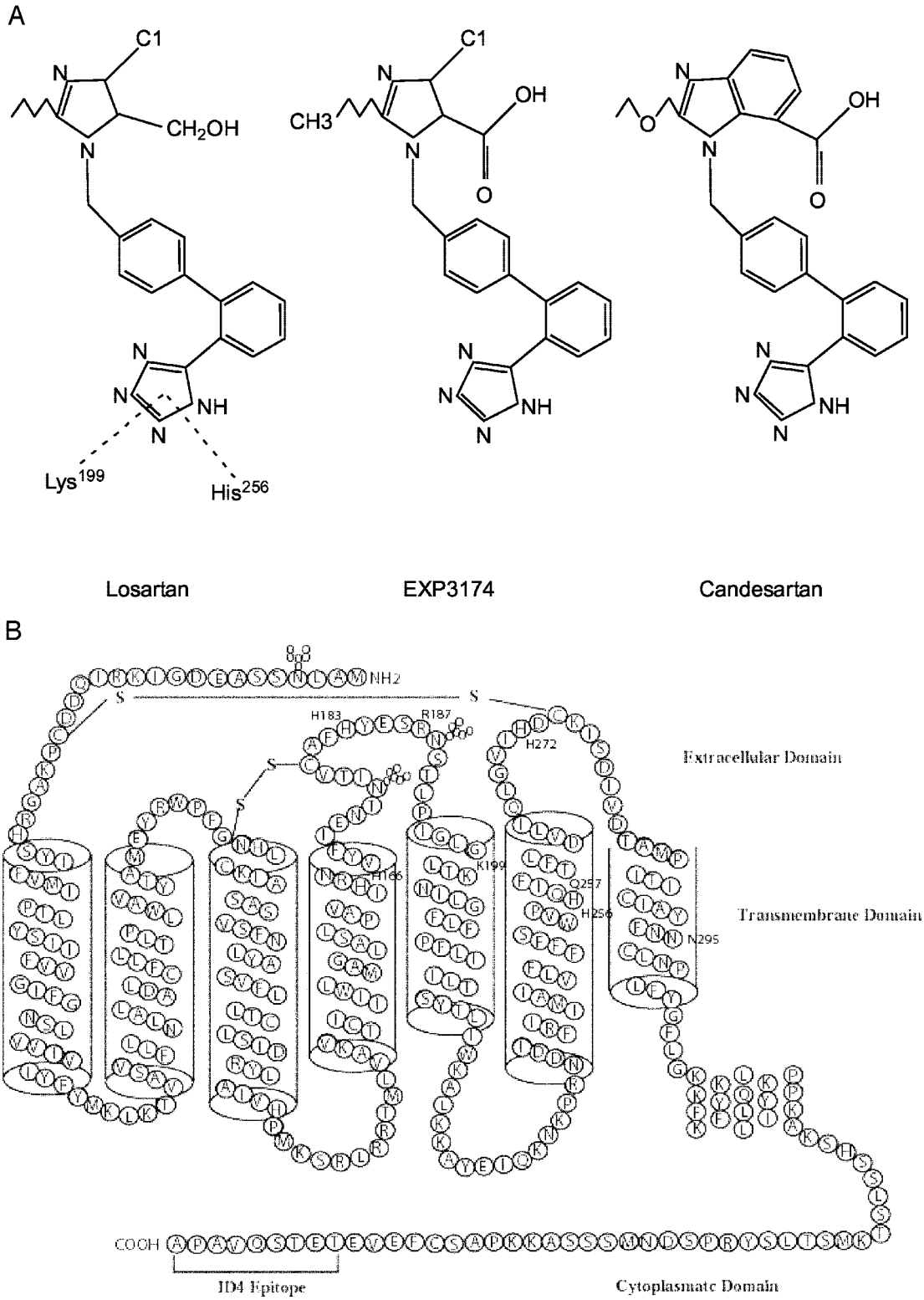

FIG. 1. **Structure of the ligands and the receptors.** *A*, chemical structure of losartan, EXP3174, and candesartan. Structure of EXP3174 contains an α-carboxylate group, and the structure of candesartan contains α-carboxylate and ethoxy groups in addition to the tetrazole group. *B*, secondary structure model of rat AT$_1$ receptor, revised based on the structure of bovine rhodopsin. Candidate residues for this study are *numbered*. The residues Lys^{199} and His^{256} have previously been shown to form ion pairs with tetrazole group of losartan. The epitope tag attached at the C-terminal end for detection by the ID4 monoclonal antibody is *underlined*. Attachment of this sequence does not alter the properties of the AT$_1$ receptor.

additional carboxylate group attached to the imidazole ring that may interact with a second positive-charged or hydrogen-bonding residue in the AT$_1$ receptor to produce insurmountable antagonism. The ethoxy group in candesartan may form an additional hydrogen bond. Together, these differences in bonding could be the basis for insurmountable antagonism. Therefore, our goal in the present study is to identify the specific bonding interaction that distinguishes insurmountable AT$_1$ receptor antagonists EXP3174 and candesartan from the surmountable antagonist losartan and examine whether abolish-

TABLE I

Binding affinity of the AT$_1$ wild-type and mutant receptor for peptide and non-peptide ligands

The values represented are the mean S.E. obtained from at least three independent experiments performed in duplicate. The chemical structures of the antagonists are listed in Fig. 1. The effect of the mutations on the binding affinity is expressed as the mutation factor, *F*mutant = K_i(mutant receptor)/K_i(WT receptor).

Receptor	K_i(*F*mutant)							
	Angiotensin II		Losartan		EXP3174		Candesartan	
	0 min	30 min	0 min	30 min	0 min	30 min	0 min	30 min
AT1WT	0.8 ± 0.1(1)	0.7 ± 0.1(0.9)	11.1 ± 1.4(1)	9.1 ± 2.5(0.8)	3.1 ± 0.4(1)	0.7 ± 0.2(0.2)	1.5 ± 0.2(1)	0.16 ± 0.03(0.1)
H166A	0.8 ± 0.1(1)	0.5 ± 0.1(0.6)	7.7 ± 2.4(0.7)	9.4 ± 4.1(0.9)	3.6 ± 0.8(1.2)	1.4 ± 0.4(0.5)	1.7 ± 0.2(1.1)	0.3 ± 0.04(0.2)
H166G	4.4 ± 1.5(5.5)	8.3 ± 4.0(10.4)	6.8 ± 1.6(0.6)	6.0 ± 1.9(0.5)	5.0 ± 1.2(1.6)	1.1 ± 0.3(0.4)	1.7 ± 0.2(1.1)	0.5 ± 0.1(0.3)
H166E	1.3 ± 0.2(1.6)	1.1 ± 0.1(1.4)	28.0 ± 5.2(2.5)	37.7 ± 5.8(3.4)	8.9 ± 0.7(2.9)	4.8 ± 1.3(1.6)	8.1 ± 2.2(5.4)	1.7 ± 0.2(1.1)
R167Q	> 800		> 14,000		> 14,000		> 14,000	
H183E	32.0 ± 8.3(40)	60.4 ± 6.0(75.5)	13.2 ± 2.3(1.2)	17.9 ± 6.9(1.6)	1.6 ± 0.1(0.5)	0.9 ± 0.1(0.3)	1.7 ± 0.2(1.1)	0.4 ± 0.2(0.3)
R187E	1.0 ± 0.2(1.3)	1.1 ± 0.2(1.4)	6.6 ± 0.7(0.6)	11.7 ± 3.4(1)	5.1 ± 1.2(1.7)	1.9 ± 0.5(0.6)	2.0 ± 0.7(1.3)	0.5 ± 0.3(0.3)
K199Q	8.8 ± 0.6(11)	14.5 ± 1.4(18.1)	17.5 ± 3.2(1.6)	6.0 ± 6.3(0.5)	6.0 ± 2.4(1.9)	2.1 ± 0.7(0.7)	8.5 ± 2.7(5.7)	2.9 ± 0.6(1.9)
H256A	0.5 ± 0(0.6)	1.6 ± 0.8(2)	11.1 ± 0.5(1)	11.4 ± 3.2(1)	4.4 ± 1.2(1.4)	1.1 ± 0.4(0.4)	5.9 ± 1.5(3.9)	0.6 ± 0.1(0.4)
Q257A	1.1 ± 0.2(1.4)	0.9 ± 0.3(1.1)	17.2 ± 4.7(1.6)	34.6 ± 7.1(3.1)	15.2 ± 1.6(4.9)	19.8 ± 1.6(6.4)	17.4 ± 1.6(11.6)	8.5 ± 2.1(5.7)
Q257E	0.4 ± 0.1(0.5)	0.4 ± 0.2(0.5)	71.4 ± 11.2(6.4)	95.6 ± 12.4(8.6)	19.0 ± 7.6(6.1)	14.4 ± 7.8(4.7)	6.8 ± 1.9(4.5)	4.2 ± 2.2(2.8)
H272G	0.5 ± 0.1(0.6)	0.9 ± 0.4(1.1)	16.7 ± 4.3(1.5)	24.4 ± 8.7(2.2)	5.6 ± 1.3(1.8)	1.2 ± 0.4(0.4)	2.0 ± 0.4(1.3)	0.4 ± 0.1(0.3)
N295A	4.0 ± 0.7(5)	4.2 ± 0.4(5.3)	913.7 ± 282.7(82)	1147 ± 246(103)	89.9 ± 11.1(29)	68.3 ± 8.9(22)	18.8 ± 8.0(12.5)	4.4 ± 0.8(2.9)
K199Q/H256A	3.6 ± 0.4(4.5)	3.7 ± 1.3(4.6)	18.9 ± 2.4(1.7)	25.3 ± 1.2(2.3)	14.8 ± 2.9(4.8)	13.1 ± 1.6(4.2)	4.1 ± 0.4(2.7)	3.5 ± 0.5(2.3)
K199Q/Q257A	1.7 ± 1.1(2.1)	1.5 ± 0(1.9)	42.7 ± 7(3.9)	31.4 ± 9.3(2.8)	19.6 ± 4.6(6.3)	18.1 ± 3.3(5.8)	13.4 ± 3.3(8.9)	10.4 ± 2.2(6.9)
H256A/Q257A	2.2 ± 0.2(2.8)	2.0 ± 0.1(2.5)	49.0 ± 8.8(4.4)	41.8 ± 11.2(3.7)	30.1 ± 3.6(9.7)	29.5 ± 5.2(9.5)	15.3 ± 2.5(10.2)	11.0 ± 1.0(7.3)
K199Q/H256A/Q257A	3.4 ± 0.4(4.3)	5.0 ± 1.9(6.3)	107.7 ± 33.6(9.7)	95.1 ± 23.1(8.6)	50.3 ± 15.8(16.2)	63.4 ± 4.8(20.5)	16.7 ± 3.4(11.1)	19.1 ± 5.8(12.7)

ing the additional interaction transforms insurmountable to surmountable antagonism.

EXPERIMENTAL PROCEDURES

Materials—Analogues of Ang II and Ang II were purchased from Bachem (Torrance, CA). ^{125}I-Labeled Ang II (specific activity, 2200 Ci/mmol) was purchased from Dr. Robert Speth (Washington State University, Pullman, WA). Losartan, [^{3}H]losartan, and EXP3174 were gifts from DuPont Merck Pharmaceutical Co. Candesartan and [^{3}H]candesartan were gifts from AstraZeneca. *myo*-[2-^{3}H(*N*)]Inositol was purchased from PerkinElmer Life Sciences. COS1 cells were obtained from American Type Culture Collection (Manassas, VA) and FUGENE 6 was purchased from Roche Diagnostics.

Mutagenesis, Expression, and Membrane Preparation—The rat AT$_1$ receptor gene (also called AT$_{1A}$) cloned in the shuttle expression vector pMT-3 was used for mutagenesis and expression as described earlier (6, 7). To express the AT$_1$ receptor protein, 10 μg of purified plasmid DNA per 10^7 cells was used in transfection. COS1 cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum were transfected by the DEAE-dextran method for membrane preparation. Transfected cells cultured for 72 h were harvested, and the nitrogen Parr-bomb disruption method in the presence of protease inhibitors prepared the cell membranes. The receptor expression was assessed in each case by immunoblot analysis (not shown) and by ^{125}I-labeled [Sar^1,Ile^8]Ang II saturation binding analysis.

Competition Binding Studies—^{125}I-Labeled [Sar^1,Ile^8]Ang II binding experiments were carried out under equilibrium conditions as previously described (6, 7). For non-preincubation competition binding experiments, membranes expressing the wild-type (WT) or the mutant receptors were incubated with binding buffer (pH 7.2, 16.3 mM Na_2HPO_4, 3.8 mM NaH_2PO_4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, and 0.2% bovine serum albumin) containing 300 pM ^{125}I-labeled [Sar^1,Ile^8]Ang II and various concentrations of Ang II or AT$_1$ receptor antagonists for 1 h at 22 °C in a 125 μl total volume. For the preincubation experiments the membranes were preequilibrated with Ang II or AT$_1$ receptor antagonists for 30 min. Nonspecific binding of the radioligand was measured in the presence of 1 μM unlabeled [Sar^1,Ile^8]Ang II. Filtering the binding mixture through Whatman GF/C glass fiber filters that were extensively washed with binding buffer without bovine serum albumin stopped the binding experiments. The bound ligand fraction was determined from the counts/min (cpm) remaining on the membrane. Binding kinetics values were determined using the computer program Ligand. The dissociation constant, $K_i = IC_{50}/(1 + (\text{ligand})/K_d)$, values given represent the mean ± S.E. of at least three independent experiments performed in duplicate.

Association and Dissociation Kinetics Studies—In the association experiments the membranes were incubated with 20 nM [^{3}H]candesartan or 50 nM [^{3}H]losartan for various time intervals between 0 and 90 min. In dissociation kinetics the membranes were incubated with 20 nM [^{3}H]candesartan or 50 nM [^{3}H]losartan for 90 min. Then 1 μM concentrations of unlabeled ligands were added, and the amount of specific binding after various time intervals between 0 and 90 min was counted. The dissociation rate constant (k_{-1}) was calculated from the first-order plot of $\ln(B_t/B_{eq})$ *versus* time, where B_{eq} and B_t are the amount of specific binding at equilibrium. The observed rate constant (K_{obs}) was calculated from the pseudo-first-order plot of $\ln(B_{eq}/(B_{eq} - B_t))$ *versus* time. The association rate constant (k_1) was calculated from slope of K_{obs} *versus* free ligand concentration in experiments with several ligand concentrations over a 10-fold ligand concentration range. The values were analyzed using GraphPad Prism.

Inositol Phosphate Formation Studies—Semiconfluent COS1 cells transfected by FuGENE 6 transfection reagent in 12-well plates were labeled for 24 h with *myo*-[2-^{3}H(*N*)]Inositol (1.5 μCi/ml; specific activity, 22 μCi/mol) at 37 °C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The labeled cells were washed 2 times with Dulbecco's modified Eagle's medium and incubated in Dulbecco's modified Eagle's medium containing 10 mM LiCl for 20 min. Ang II was then added, and incubation was continued for an additional 40 min at 37 °C. In the antagonist preincubation experiments, cells were preincubated with vehicle or antagonists for 30 min, Ang II was then added, and incubation was continued for another 40 min at 37 °C. At the end of the incubation the medium was removed, and the total soluble IP was extracted from the cells by perchloric acid extraction, as described previously (6, 7). EC_{50} and IC_{50} values were calculated by non-linear regression analysis by Graphpad Prism. The values represented were mean ± S.E. of at least three independent experiments performed in duplicate.

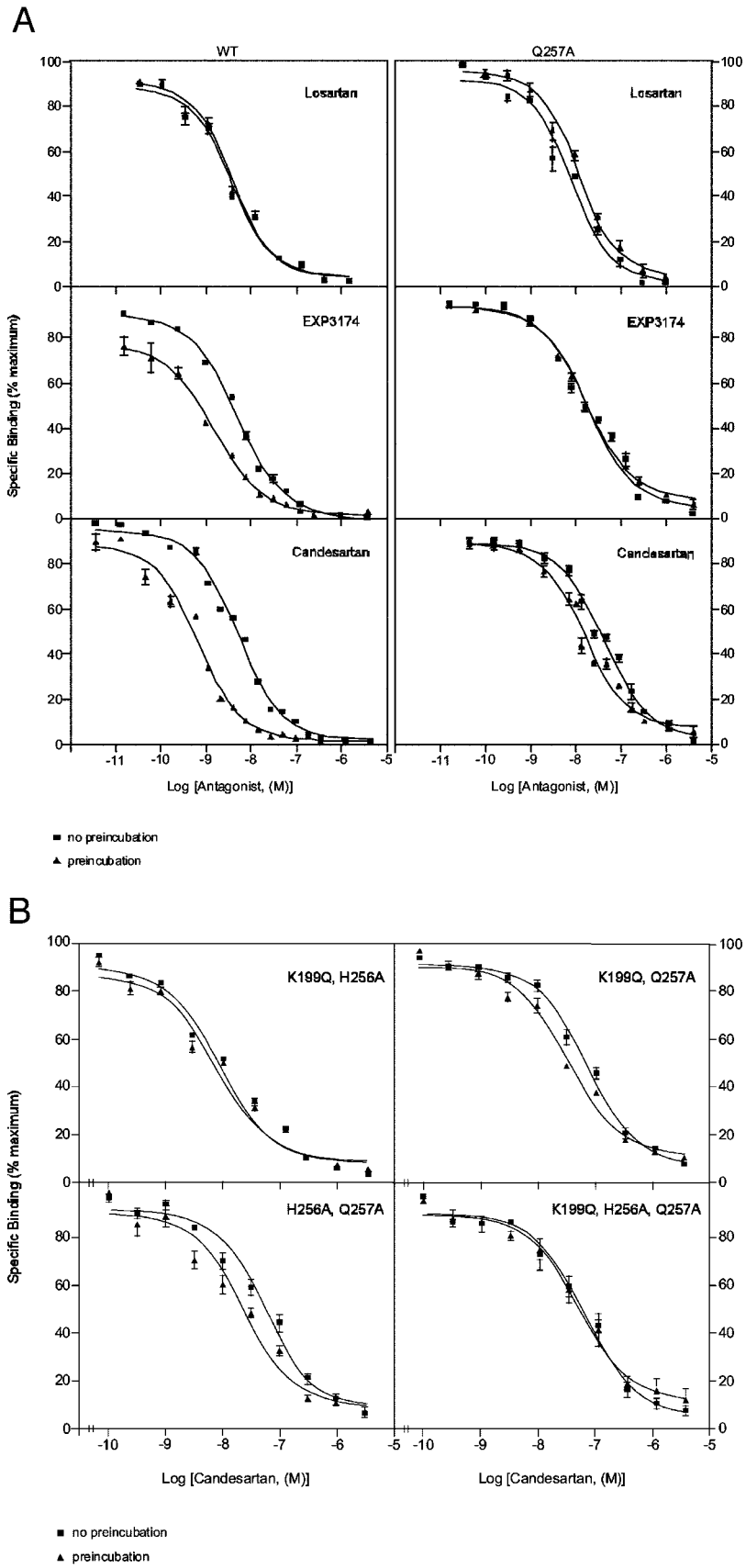

FIG. 2. **Preincubation of insurmountable antagonists increases the affinity for the WT but not the Q257A mutant AT_1 receptor.** COS1 cell membranes expressing WT and Q257A mutant (*panel A*) or the specified double and triple mutants (*panel B*) were used in competition binding experiments with (▲) and without (■) preincubation with the specified non-peptide antagonist in each case. The ^{125}I-labeled [Sar^{1}Ile^8]Ang II specific binding value at each antagonist concentration was then normalized to a percentage of maximum specific binding in the absence of any antagonist in each experiment. Data are the mean ± S.E. obtained from at least three independent experiments.

Molecular Modeling of the AT_1 Receptor—The molecular model used the bovine rhodopsin structure (Protein Data Bank code 1F88) as a template (8). Using the ClustalW alignment program (Julie D. Thompson, Desmond G. Higgins, and Toby J. Gibson, European Bioinformatics Institute, Cambridge, UK), we performed the alignment of rat AT_{1A} receptor primary sequence with the bovine rhodopsin primary sequence. The amino acid mutations were performed by Swiss-PdbViewer (9) followed by energy minimization using the Gromacs molecular dynamics simulation program (10). During the entire modeling process the secondary structure was monitored to ensure that the AT_1

secondary structure was conserved. This model was used for docking both the agonist and antagonist ligands, performed using AutoDock 3.0.5 program (27). The procedure uses a Monte Carlo simulated annealing or a genetic algorithm technique for configurational exploration with rapid energy evaluation using grid-based molecular affinity potentials.

RESULTS

Candidate Residues Specific for Insurmountable Binding of the Ligands—The competitive binding affinity of Ang II, losartan, EXP3174, and candesartan for the WT and various mutant AT_1 receptors are shown in Table I. The affinity of Ang II decreased in H166G, R167Q, H183E, K199Q, and N295A mutants and was not significantly altered in H166A, H166E, R187E, H256A, Q257A, Q257E, and H272G mutants. The K_i value of the WT and all of the mutants for Ang II did not significantly change upon preincubation with competitive ligand, *i.e.* the unlabeled Ang II mixed with the membrane 30 min before the addition of ^{125}I-labeled [Sar1,Ile8]Ang II in the competitive ligand binding experiments. Preincubation did not change the K_i of losartan for the WT and 12 single residue mutants, although the K_i itself was altered in H166E, R167Q, Q257E, and N295A mutants. The affinity of EXP3174 was reduced in H166E, R167Q, Q257A, Q257E, and N295A mutants. However, preincubation of EXP3174 with the WT decreased the K_i 4.4 times compared with no preincubation (adding the competing and tracer ligands together). A decreased K_i for EXP3174 upon preincubation was observed in all mutants except the Q257A and Q257E mutants. The affinity of candesartan was reduced in H166E, R167Q, K199Q, H256A, Q257A, Q257E, and N295A mutants. A 10-fold decrease in the K_i of candesartan for the WT receptor was measured upon preincubation. The preincubation-induced decrease of K_i was 3–10-fold in most mutants. However, in the Q257A and Q257E mutants, the decrease of K_i was 2- and 1.6-fold, respectively.

To unravel putative interaction between residues involved in binding the surmountable antagonist losartan and the insurmountable antagonists, EXP3174 and candesartan, three double mutants and one triple mutant of the AT_1 receptor were examined (Table I). The combined effect of double mutations K199Q/H256A, K199Q/Q257A, and the H256A/Q257A on the K_i of losartan, EXP3174, and candesartan was nearly additive in comparison to each of the single mutations. The overall effect on K_i in the double mutants was nearly equal to the combined change of component single mutations. Deviation from additivity observed reflects the extent to which disruption of interaction between Lys^{199}, His^{256}, and Gln^{257} changes measured K_i. Combining all three mutations reduced the affinity >10-fold for all 3 antagonists. The affinity increase for candesartan observed upon preincubation was abolished in all four mutants. The affinity-shift for EXP3174 was also abolished in these mutants. Altogether, effects of these mutations suggest that a combinatorial interaction of residues Lys^{199}, His^{256}, and Gln^{257} is also necessary for insurmountable binding of antagonists.

Effect of the Q257A Mutation on Losartan, EXP3174, and Candesartan Binding Kinetics—Fig. 2*A* depicts the effect of preincubation on competition binding of surmountable and insurmountable antagonists. Preincubation did not influence the losartan binding isotherm in the WT and the Q257A mutant. The binding isotherms for the insurmountable antagonists were shifted left in the WT. In the Q257A single mutant, the EXP3174 curve did not shift upon preincubation, and the magnitude of the candesartan curve-shift was significantly smaller compared with that in the WT AT_1 receptor. Candesartan binding isotherm-shift induced by pre-equilibration with the drug was completely abolished in the double and triple mutants (Fig. 2*B*).

Direct binding experiments suggest that the dissociation of the insurmountable antagonist, candesartan, from the bound receptor was slower than the dissociation of structurally analogous surmountable antagonist, losartan. The association and dissociation kinetics of [^{3}H]losartan and [^{3}H]candesartan for the WT and the Q257A mutant AT_1 receptor were compared (Fig. 3). We did not analyze EXP3174 in this set of experiments because [^{3}H]EXP3174 was not available to use. The observed rate of association (K_1 min^{-1} nM^{-1}) of ^{3}H-candesartan with WT (0.0105 ± 0.0004) was 2.6-fold more than the rate of association of [3H]losartan with the WT (0.0271 ± 0.0002). The dissociation rate (K_{-1} min^{-1}) of [3H]candesartan with WT (0.0108 ± 0.0044) was 55-fold lower than the dissociation rate of [3H]losartan with the WT (K_{-1} min^{-1}, 0.595 ± 0.199). The Q257A mutation affected both association and dissociation rates of candesartan (Fig. 3). The [3H]candesartan association rate (0.00054 ± 00009) for this mutant was reduced 21-fold and the dissociation rate (0.0313 ± 0.002) was increased 2.9-fold when compared with the WT. Association of [3H]losartan with the Q257A mutant (0.0029 ± 0.0001) was 9.3-fold slower. The dissociation rate of [^{3}H]losartan with the WT (K_{-1} min^{-1}, 0.595 ± 0.199) and Q257A mutant (0.474 ± 0.026) were nearly comparable. The K_i values of WT calculated from K_{-1} and K_1 for losartan (21.9 ± 7.2 nM) and candesartan (1.04 ± 0.46 nM) were similar to the K_i values calculated from competition binding experiments (Table I). The K_i values of Q257A mutant calculated from K_{-1} and K_1 for both losartan (166.0 ± 6.3 nM) and candesartan (68.9 ± 6.2 nM) were, respectively, 10- and 4-fold higher than K_i values estimated from competition binding experiment in (Table I). This discrepancy, seen only in the Q257A mutant for both ligands, may be a reflection of a disproportionate effect of mutation on association rate than dissociation rate.

Antagonism Pattern of Ang II-induced IP Response in the WT and Q257A Mutant AT_1 Receptors—The pattern of antagonism of Ang II concentration-response in the WT and Q257A mutant suggests that display of insurmountable behavior by EXP3174 and candesartan requires the Gln^{257} side chain in the WT receptor (Fig. 4). The EC_{50} value was 0.005 ± 0.001 nM for the Ang II-induced IP production in the WT AT_1 receptor-transfected cells. Pre-equilibration with insurmountable antagonists for 30 min before Ang II addition caused a rightward shift in the response curves. The EC_{50} values were 12.6 ± 1.4 nM with 10 nM EXP3174 and 34.3 ± 4.9 nM with 10 nM candesartan. The maximal response to Ang II decreased for both EXP3174 (77.7 ± 4.3%) and candesartan (47.5 ± 5.4%). In contrast, 100 nM losartan produced a rightward shift (EC_{50}, 3.6 ± 0.8 nM) but did not decrease the maximal response. In the Q257A mutant transfected cells, EXP3174 and candesartan only produced a rightward shift without altering the maximal response (see Fig. 4). The EC_{50} values were 0.23 ± 0.11 nM with the vehicle, 1.5 ± 0.3 nM with 100 nM losartan, 4.6 ± 1.3 nM with 10 nM EXP3174, 15.4 ± 1.8 nM with 10 nM candesartan. The maximal IP responses were 96.6 ± 2% in the presence of 100 nM losartan, 96.1 ± 2% in the presence of 10 nM EXP3174, and 93.3 ± 3% in the presence of 10 nM candesartan. The transfection conditions were adjusted such that the cell surface B_{max} values of the WT and mutant receptors obtained were comparable in all of the experiments.

Antagonism of WT and Q257A Mutant AT_1 Receptor Activation—The activation of WT and Q257A AT_1 receptor inhibited by antagonists in a concentration-dependent manner is shown in Fig. 5. The intrinsic basal activity of both receptors was inhibited by all three antagonists, suggesting that their inverse agonist activity is independent of insurmountable antagonist activity (Fig. 5*A*). The order of potency of the antagonists was candesartan (IC_{50} = 8.4 ± 2.2 *versus* 77.1 ± 11.9 nM, respec-

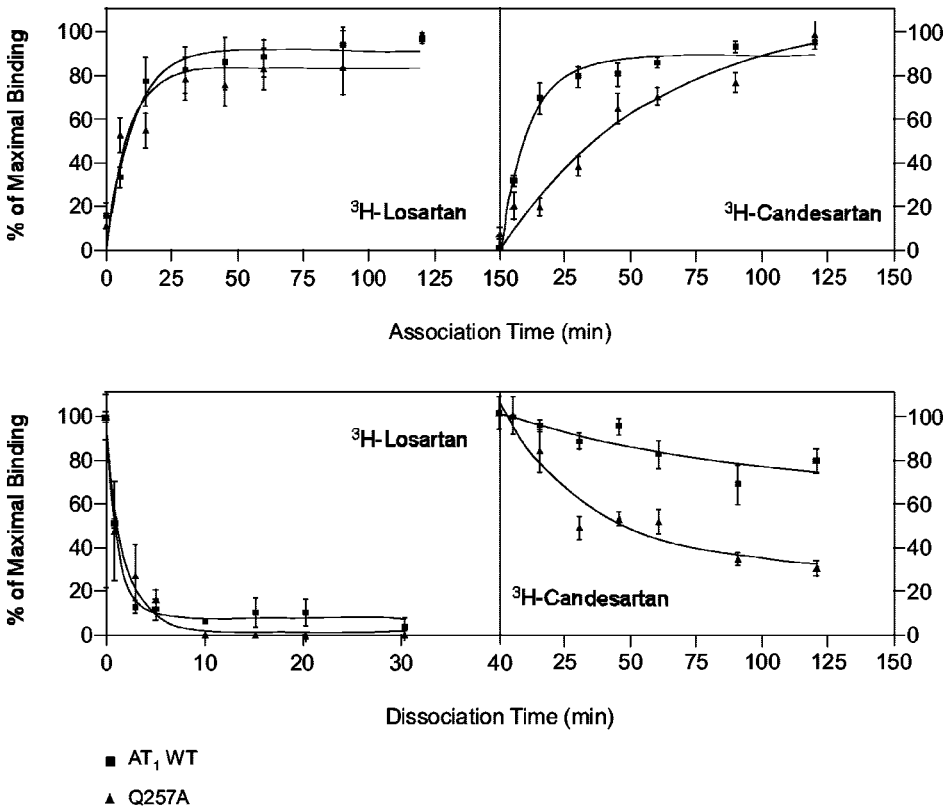

FIG. 3. **Association of the insurmountable antagonist is faster and the dissociation is slower in the WT than the Q257A mutant AT_1 receptor.** Time-course of direct association and dissociation kinetics of [^{3}H]losartan and [^{3}H]candesartan binding to AT_1 WT and Q257A mutant receptor. In dissociation experiments, 50 nM [3H]losartan was mixed with COS1 membranes after equilibrium was reached; dissociation was induced by the addition of unlabeled 1 μM losartan. In the dissociation kinetics of [^{3}H]candesartan, after 20 nM [^{3}H]candesartan mixed with membranes had reached equilibrium, dissociation was induced by the addition of unlabeled 1 μM candesartan. The specific binding is calculated as a percentage of the specific binding at equilibrium. Data are the mean ± S.E. obtained from at least three independent experiments. The association experiments shown here were done under identical conditions. However, the association rate (K_1) of the antagonists derived was not useful for any interpretation because the calculated K_1 values were negative. Therefore, the association experiments were repeated for both WT and Q257A mutants with [3H]losartan and [^{3}H]candesartan over a 10-fold concentration range. This allowed us to calculate the association rate constant from the slope of a plot of K_{obs} *versus* free ligand concentration. The values are given under "Results."

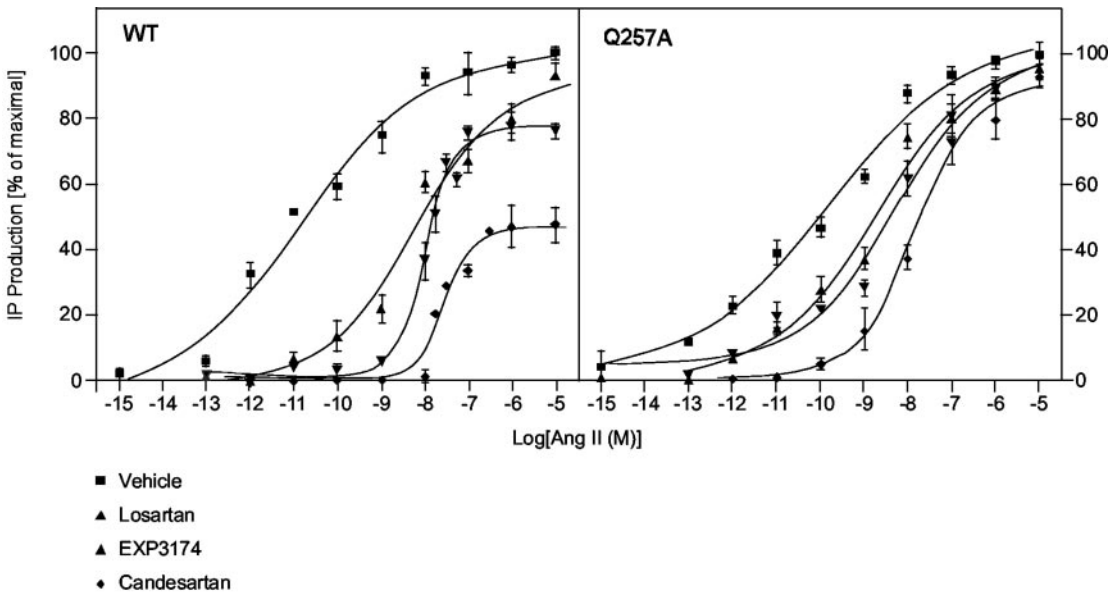

FIG. 4. **Candesartan and EXP3174 fail to exert insurmountable antagonism of the Ang II-induced IP production in the Q257A mutant AT_1 receptor.** Ang II-mediated IP response curves in COS1 cells transfected with WT and Q257A mutant AT_1 receptor are shown. The cells were preincubated for 30 min with 100 nM losartan or 10 nM EXP3174 or 10 nM candesartan before stimulation with Ang II.

tively) > EXP3174 (IC_{50} = 30.3 ± 8.1 *versus* 523.0 ± 36.3 nM, respectively) > losartan (IC_{50} = 128.4 ± 21.5 *versus* 771.1 ± 56.7 nM, respectively) for both WT and Q257A mutant AT_1-transfected cells. All of the antagonists displayed higher IC_{50} values with the Q257A mutant compared with WT. It appears that all three antagonists are capable of achieving nearly sim-

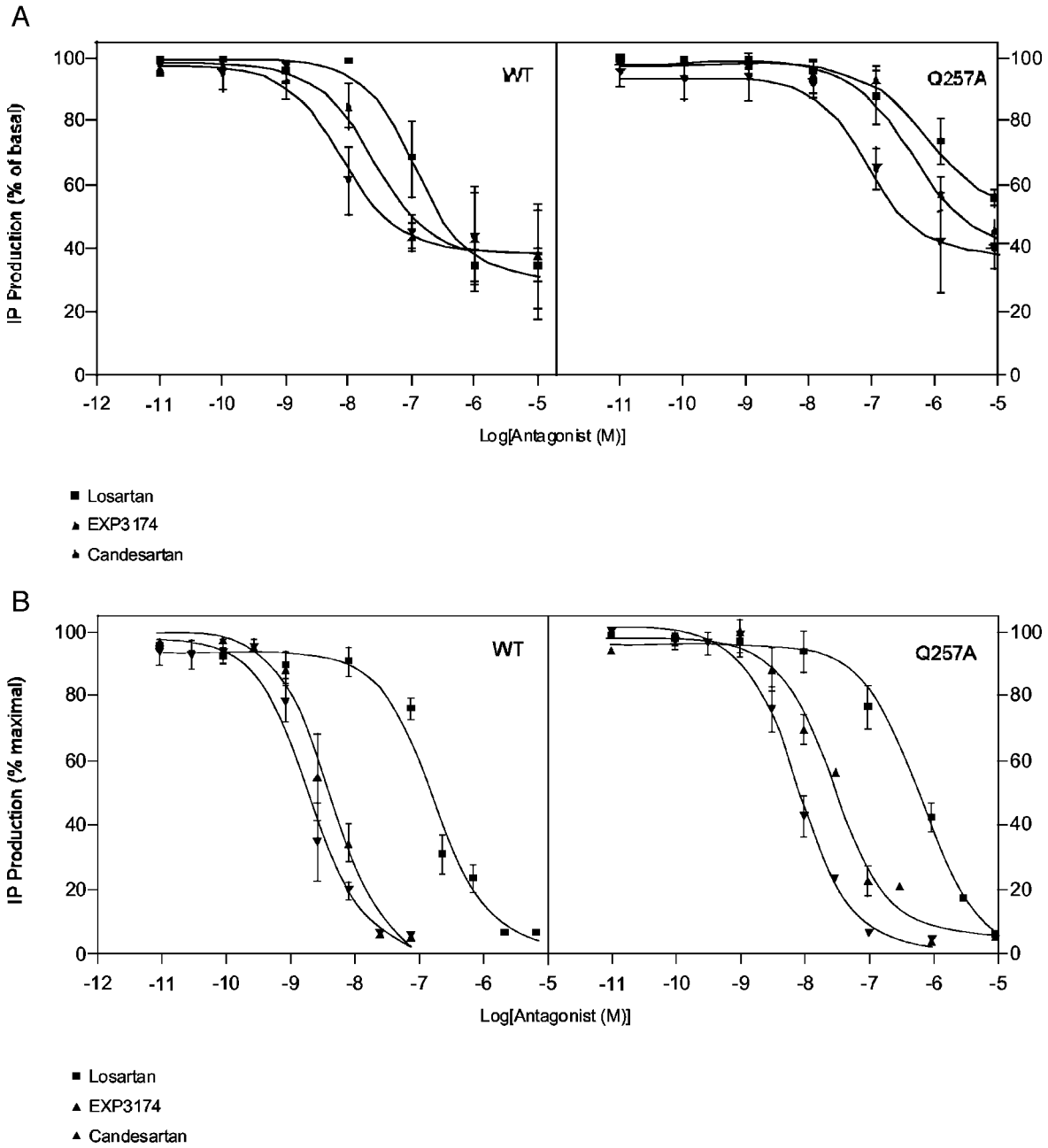

FIG. 5. **Inverse agonist activity of losartan, candesartan, and EXP3174 is unaffected in inhibiting IP production induced by WT and Q257A mutant AT_1 receptor.** *A*, concentration-dependent inhibition of the agonist-independent basal activity after antagonist preincubation of COS1 cells transfected with WT and Q257A mutant AT_1 receptor for 30 min. The IP accumulation is expressed as percent of basal IP accumulation with vehicle alone. The B_{max} values estimated per mg of total protein are as follows: WT, 10.1 ± 1.9 pmol/mg; Q257A, 4.1 ± 0.5 pmol/mg. Data are the mean ± S.E. obtained from three independent experiments. *B*, inhibition of Ang II-induced IP production. Cells were preincubated for 30 min with a range of concentration of losartan, EXP3174, or candesartan. The IP accumulation is expressed as percentage of maximum Ang II-dependent increase (8000 ± 1000 cpm/pmol receptor) over the basal values obtained after treatment with vehicle (~1500 ± 300 cpm/pmol of receptor). Data are the mean ± S.E. obtained from at least three independent experiments.

ilar maximum inhibition in both receptors.

The same pattern of antagonism was observed in Ang II-induced activation experiments (Fig. 5*B*). The order of potency of the antagonists was candesartan (IC_{50} = 2.9 ± 0.5 *versus* 7.9 ± 1.4 nM, respectively) > EXP3174 (IC_{50} = 5.0 ± 1.2 *versus* 32.0 ± 6.7 nM, respectively) > losartan (IC_{50} = 277.0 ± 42.7 *versus* 545.7 ± 134.6 nM, respectively) for both AT_1 WT and Q257A mutant-transfected cells. EXP3174 produced the largest rightward shift in the mutant.

Molecular Model of the Candesartan-AT_1 Receptor Complex—A representation of the candesartan-AT_1 receptor complex is shown in Fig. 6*A*. The free energy of binding was −92 kcal/mol for the WT-candesartan complex. The free energies were −5 to −13 kcal/mol for the Q257A-candesartan, the Q257A-EXP3174, and the WT-losartan complexes, suggesting that the binding free energies for antagonist-receptor complexes in the surmountable state are nearly comparable with each other and significantly differ from the complex in the insurmountable state. The final internal energy of the ligands were −0.49 to +0.47 kcal/mol, implying that the conformation of the ligands are not strained. With this model we aimed to investigate the relationships between the three residues, Lys^{199}, His^{256}, and Gln^{257}, which were experimentally determined to contribute to the effects of insurmountable antago-

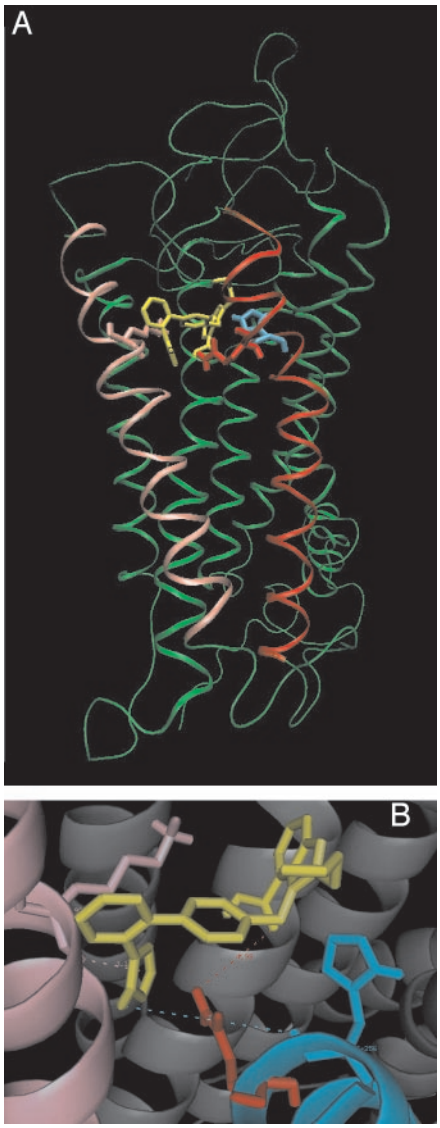

FIG. 6. **Molecular modeling of AT$_1$ receptor.** *A*, model of rat AT$_{1A}$ receptor based on high resolution structure of bovine rhodopsin. The Cα-trace of the entire receptor viewed from the side, color-coded green except for TM-5 (*pink*) and TM6 (*red*). *B*, candesartan (*yellow*), location in the pocket predicted by AutoDock Computer Program is shown. Residues shown are Lys^{199} (*pink*), His^{256} (*blue*), and Glu^{257} (*red*). Modeling reveals a tight network of these and other side chains.

nists. The relative positions of these three residues in three-dimensional space is elucidated (Fig. 6*B*).

In this conformation the distance between and α-COO$^-$ of candesartan was 4.98 Å, and the distance between Ala^{257} and α-COO$^-$ of candesartan was 8.18 Å. The distances between the tetrazole group of candesartan and the side chains of Lys^{199} and His^{256} were determined to range from 2.51 to 7.31 Å (Fig. 6*B*). In addition several neighboring aromatic and hydrophobic side chains contribute to a cluster of interactions with the biphenyl core. In the absence of candesartan, the Lys^{199}, His^{256}, and Gln^{257} were 3–6 Å apart, allowing them to be involved in a network of weak interactions.

DISCUSSION

The most important result of this study is the finding that the Q257A mutation renders the AT$_1$ receptor insensitive to insurmountable antagonism by EXP3174 and candesartan. Preincubation of these insurmountable antagonists with the WT receptor leads to apparent increase of binding affinity and suppression of the Ang II-induced maximal IP response. Both of these effects exerted by EXP3174 were nearly completely abolished, and the effects exerted by candesartan were suppressed in the Q257A mutant (as seen in Figs. 2–4). It is important to note that the classical antagonist and inverse-agonist activities of EXP3174, candesartan, and losartan were not affected (as seen in Fig. 5) by the Q257A mutation. Thus, display of insurmountable antagonism appears to specifically require the Gln^{257} side chain in the WT AT$_1$ receptor and the α-carboxyl group in candesartan and EXP3174 (Fig. 1). Interaction between them appears to be a hydrogen bonding since substitution with potentially negative-charged Glu side chain did not cause a greater defect than substitution with Ala, implying that the carboxyl group of the substituted Glu may be protonated, allowing it to form a hydrogen bond with EXP3174 and candesartan. This unique property was not observed in several other AT$_1$ receptor mutants of residues that were considered candidates (Fig. 1) to interact with this α-carboxyl group of the insurmountable antagonists based on a molecular model (Fig. 6). Preincubation of each mutant receptor with candesartan and EXP3174 led to increases of affinity as in the WT (Table I). Previously, Fierens *et al.* (11) reported that a K199Q mutation was more detrimental for the binding of the insurmountable antagonists than the binding of surmountable antagonists, and Schambye *et al.* (12) report that a N295A mutation selectively reduced the affinity of insurmountable antagonists. Although the substitution of Lys^{199} decreased the affinity for candesartan more than losartan, this effect was not selective since the EXP3174 binding was not effected in a similar way (see Table I). The K199Q/H256A double mutant decreased the affinity for all three antagonists, implying that the His^{256} side chain compensates the effect of Lys^{199} mutation to varying degrees. This observation is consistent with a previous report from this laboratory (6) in which Lys^{199} and His^{256} were proposed to interact with the tetrazole group that is common to all three antagonists. Substitution of Asn^{295} with Ala on the other hand decreased affinity for all ligands including Ang II and losartan, suggesting that Asn^{295} interaction is not specific for insurmountable antagonists. Instead, mutation of this residue likely affects general conformation of the TM domain, accounting for loss of affinity for all ligands. Based on these observations we conclude that hydrogen bonding with the Gln^{257} side chain is the molecular basis for insurmountable behavior of EXP3174.

A residual insurmountable effect of candesartan on the Q257A mutant (2-fold increase in affinity for this mutant compared with 10-fold in WT in preincubation experiment) is likely due to the interaction of its ethoxy group with an unknown site on the AT$_1$ receptor, which distinguishes it from EXP3174. Thus, additive-bonding interactions may account for different insurmountable efficacy of AT$_1$ antagonists reported (2–5). The finding further substantiates the observations that mutations at sites not critical for insurmountable antagonism, such as Lys^{199} and His^{256}, can indirectly affect interaction with Gln^{257} (Fig. 2*B*). Thus, insurmountable binding can change to surmountable binding from not achieving the critical Gln^{257} antagonist contact due to additive entropy effects of multiple mutations.

On the basis of the finding that the insurmountable antagonists behave like surmountable antagonists with the Q257A mutation, we propose that in the WT receptor an antagonist-mediated conformational transition is responsible for both slow dissociation and inhibition of maximal IP response. In the past both equilibrium and kinetic models of insurmountable antagonism of the AT$_1$ receptor have argued that the resulting "hemi-equilibrium" pattern of the diminished functional response (see Fig. 4) would be dependent on the antagonist dis-

sociation rate (2–5). The magnitude of the response to agonist will depend on the rate and the degree of activation. This implies that the maximum response to Ang II after preincubation with an insurmountable antagonist can be recovered simply by increasing the contact time of Ang II. But under our experimental conditions, *i.e.* 45 min of exposure of up to 10,000-fold molar excess of Ang II did not restore the response of WT inhibited by candesartan and EXP3174 (Fig. 4). Our findings are consistent with previous reports in both aortic contraction assays and transfected cells in which time for resensitization to Ang II was estimated to be >2 h. The reduced dissociation and association rate constants in the Q257A mutant suggest that this mutation affects formation of the slow-dissociating insurmountable antagonist-receptor complex. The barrier for dissociation of EXP3174 and candesartan in the WT receptor is gained by their specific interaction with the Gln^{257} side chain.

An alternative explanation could be that the "slow-dissociating" antagonist-receptor complexes are in a different subcellular location. The peptide antagonists of the AT_1 receptor are capable of inducing AT_1 receptor internalization (13); therefore, the possibility of candesartan-induced internalization was previously suggested (14). Partitioning of antagonist-AT_1 receptor complex into an acid-resistant subcellular compartment was reported (15). But in internalization-defective AT_1 mutant the candesartan receptor complex was still found to be acid-resistant (16). Direct internalization measurements with enhanced green fluorescent protein-tagged AT_1 receptor treated with losartan or candesartan suggest the absence of internalization to intracellular vesicular compartments (17, 18). These findings suggest that insurmountable antagonists are unlikely to promote internalization similar to that induced by peptide antagonists and agonists. Either the mild acid treatment was insufficient to discriminate between surface bound and a novel form of antagonist-receptor complex or acid resistance is a conformational state of the candesartan-bound AT_1 receptor in the plasma membrane. An insurmountable antagonist-induced conformation change is necessary to consider either of these explanations. The evidence presented here suggests that the Q257A mutation must affect this step in the AT_1 receptor.

Insurmountable Antagonist Versus Inverse Agonist—With respect to the potential involvement of Gln^{257} bonding with candesartan or EXP3174 in inverse agonist properties of these compounds, data presented in Fig. 5 suggest that the molecular basis of insurmountable antagonist and inverse agonist phenomenon are different. We conclude that the Gln^{257}-candesartan (EXP3174) bond is not directly involved in mediating the conformation that results in the inactivation of the AT_1 receptor function. The bond sustains the receptor in the inactive conformation and prevents access to agonist. For both basic pharmacology and drug design it is important to understand this distinction in mechanisms whereby drugs achieve distinct effects. It has been assumed that these drugs achieved their effects by stabilizing an inactive state of the receptor at the expense of partially (R′)- or fully (R*)-activated states (19, 20). We suggest that the insurmountable AT_1 receptor antagonists reveal an additional step that should be considered in the models of antagonism of GPCRs. Thus, in addition to becoming an important therapeutic class of drugs, the AT_1 receptor antagonists are exciting from a receptor theory viewpoint as well.

Model and General Implications—Mechanisms of how the binding of agonists and inverse agonists preferentially stabilize, respectively, the active and inactive conformations of GPCRs requires better understanding at a molecular level. The current view is that GPCRs exist in a dynamic equilibrium between active (R*) and inactive states governed by an allosteric constant (R*), and the efficacy of a ligand is a function of its relative affinity for R and R* states. Classical antagonists, also called neutral antagonists, have equal affinity for both receptor states, inverse agonists preferentially bind to R, thereby shifting the position of equilibrium toward the inactive state, and the agonists preferentially bind to R*. There is rapid progress in understanding the molecular mechanisms of agonist-mediated activation, but the mechanisms by which the inactive state(s) of the receptor is generated by inverse agonist binding remain unclear. The Gln^{257} mutations reported in this study shed new light on this issue.

The prototypical GPCR, mammalian rhodopsin, exists in at least three different states, the silent "ground" state (Rg) is stabilized by the inverse agonist, 11-*cis*-retinal; the low activity state is the retinal-free opsin; and the fully active state (R*) is all-*trans*-retinal bound metarhodopsin II (20–22). Stabilization of the Rg state by the 11-*cis*-retinal lowers the activity 6–10 times, which enables rhodopsin to operate as a single photon detector even in the presence of high levels of activated rhodopsin in rod cells (22). The 11-*cis*-retinal should be considered an insurmountable antagonist in addition to inverse agonist of opsin since the K_d of the 11-*cis*-retinal complex with opsin is >150 h in the dark. The stability of the Rg state of opsin is due to a covalent protonated Schiff base linkage with Lys^{296} in the center of the TM domain, which is stabilized by the Glu^{113} counter ion and a network of hydrogen bonds. As to the ligand-receptor interactions in other GPCRs, the antagonists, full or partial agonists were found to mediate different conformational changes in the β_2-adrenoreceptor (23, 24). Therefore, it seems likely that different activity states are a heterogeneous population of receptor conformations. How the stability of new states in GPCRs is achieved in the face of interaction of different types of ligands is not clear. Neighborhood and environment effects on broad ensembles of microscopic conformations of GPCRs need to be better understood.

Our observations with the Gln^{257} mutants of the AT_1 receptor, which completely blocked insurmountable antagonist activity while maintaining specific binding, the inverse agonist effects, and the agonist-mediated transition to the R* conformation, suggest that an "inactive state" comparable but not identical to the Rg state of opsin should be considered. It is now well established that activation of GPCRs involves disruption of a network of intramolecular interactions leading to separation of TM-III and TM-VI helices (20). Disruption of a network of interactions that stabilize the receptors in inactive conformations constitutes the receptor activation switch. The precise residues involved in these interactions may vary between different receptors (20). The data presented in this study suggest that a different network of intramolecular interactions in the AT_1 receptor underlies the actions of insurmountable antagonists. The structural model of the AT_1 receptor, presented in Fig. 6,[2] indicates that the side chains of residues Lys^{199}, His^{256}, and Gln^{257} are 3–6 Å apart, and the aromatic cluster formed between the side chains of Phe^{259} and Trp^{253}, features conserved in other GPCRs, also constitute a constraining network. Stabilization of the biphenylimidazole antagonist-bound Rg state likely involves this network. We propose that AT_1 receptor insurmountable antagonist binding requires "leaning" on the Gln^{257} residue in addition to the interaction of core structure of antagonists with the above-mentioned network. The leaning ability conferred by the presence of the α-carboxyl group in EXP3174 and Candesatran is the key for functional specialties (*i.e.* insurmountable antagonism in this case) of pharmacological derivatives and some times drug metabolites (*i.e.* EXP3174 is a metabolite of losartan) (25). It is worth

[2] C. Gogonea and S. Karnik, unpublished.

noting here that leaning behavior of compounds could explain accidental antagonist to agonist transformation during systematic drug development efforts. Indeed, a subset of substituted biphenylimidazole derivatives (*e.g.* L-162,313) was found to possess agonist properties at the AT_1 receptor (26). Further studies may reveal the existence of constraining networks and leaning behavior in other GPCR antagonists. Elucidation of such networks could provide a better understanding of the effects of insurmountable antagonists, inverse agonists, and antagonists on the conversion between different conformations of the GPCR structure.

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