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Reversible and Syntopic Interaction between Angiotensin Receptor Antagonists on Chinese Hamster Ovary Cells Expressing Human Angiotensin II Type 1 Receptors

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ABSTRACT. Evidence for a competitive type of interaction between angiotensin II type 1 (AT_1) antagonists on Chinese hamster ovary cells expressing the human AT_1 receptor (CHO-AT_1) was obtained by analyzing the binding of [^{3}H]-2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid ([^{3}H]candesartan) and by measuring the AT-induced production of inositol phosphates. The AT_1 antagonists candesartan, 2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (EXP3174), or 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan) produced a concentration-dependent increase in the apparent K_d values of [^{3}H]candesartan in saturation binding experiments, while the B_{max} values were unchanged. Furthermore, the dissociation rate of the radioligand initiated by 1 μM unlabelled candesartan was not changed in the presence of 10 μM losartan, 10 μM EXP3174, or 10 μM irbesartan (2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]2-imidazolin-5-one)). Preincubation of the CHO-AT_1 cells with candesartan, EXP3174, and irbesartan caused a reduction in the maximal AT-induced inositol mono-, bis-, and trisphosphate production. This insurmountable effect was reversed in the presence of 1 μM losartan. In line with this finding, the insurmountable antagonist concentration–inhibition curves at 10 μM AT were shifted to the right in the presence of losartan. For candesartan this effect was concentration-dependent, yielding a pK_B value for losartan of 7.7, which is similar to the pK_B from previously obtained AT concentration–response curves. Finally, the dissociation rate of candesartan, EXP3174, irbesartan, and losartan was determined by measuring the recovery of AT responses after antagonist pretreatment and washing of the cells with medium containing 1 μM losartan to prevent re-association of the insurmountable antagonists. In addition, similar kinetic data were obtained from the slowing of the [^{3}H]candesartan association rate to antagonist preincubated cells. BIOCHEM PHARMACOL **59**;8:927–935, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. AT_1 antagonists; CHO cells; inositol phosphate; candesartan; irbesartan; EXP3174; losartan; surmountable; insurmountable

During the last decade, much interest has been devoted to the development of non-peptide AT_1† receptor antagonists for the clinical treatment of hypertension and congestive heart failure [1, 2]. The *in vitro* pharmacology of these antagonists has been studied by measuring AT-induced contraction of rabbit aorta rings/strips as well as IP production in cell lines expressing transfected AT_1 receptors [3–6], neither system having a detectable receptor reserve. These studies revealed the existence of two categories of antagonists. Surmountable antagonists, such as losartan, produced parallel rightward shifts of the AT concentration–response curve, without affecting the maximal response [4, 7, 8]. Insurmountable antagonists also depressed the maximal response to AT to a degree that varied from one antagonist to another. This may range from a partial decline for the antagonists irbesartan, valsartan, and EXP3174 (the active metabolite of losartan) to an almost complete effect for antagonists such as GR117289 and candesartan [4, 8, 9–13].

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† *Abbreviations:* AT, angiotensin II; AT_1, AT receptor type 1; candesartan, 2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid; CHO-AT_1 cells, Chinese hamster ovary cells expressing human AT_1 receptors; DMEM, Dulbecco's modified Eagle's medium; EXP3174, 2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid; IP, inositol mono-, bis-, and trisphosphates; irbesartan, 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]2-imidazolin-5-one); losartan, 2-*n*-butyl4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole; and k_{obs}, pseudo-first-order association rate constant.

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The ability of AT_1 receptor antagonists to depress the maximal response to AT was dependent on the experimental conditions. In this respect, it was noticed by Criscione *et al.* [11] that valsartan, an insurmountable antagonist in aortic ring contraction studies, was unable to depress the maximal AT-induced aldosterone release in bovine adrenal glomerulosa cells. These experiments were done under co-incubation conditions as opposed to the routine procedure for aortic strip contraction studies which involved the pre-exposure of the receptors to the antagonist. Similarly, co-incubation with candesartan, EXP3174, and irbesartan only produced parallel rightward shifts of the concentration–effect curves of AT-mediated IP production in CHO-AT_1 cells [6]. In the same line, comparative radioligand binding studies revealed that antagonists such as candesartan, SRL1080277, sarile, and UR-7280 decreased the maximal binding capacity of labeled AT when added to the receptors before the radioligand and that the same antagonists did not affect the maximal binding under co-incubation conditions [14–16]. Based on these findings, it was proposed that insurmountable AT_1 receptor antagonists inhibit the response to AT in a competitive fashion in co-incubation experiments but that, when pre-exposed to the receptor, their antagonistic action may be so slowly reversible that it cannot be overcome during the ensuing short exposure of the receptors to AT [6]. A link between the insurmountable nature of certain AT_1 receptor antagonists and their long-lasting action was also suggested in several other studies [16–21]. Surmountable antagonists, on the other hand, were also competitive, but their antagonistic action was readily reversible so that it could be completely overcome upon a subsequent exposure of the tissue or cells to the agonist.

The aim of the present study was to determine whether insurmountable and surmountable AT_1 antagonists are mutually competitive. Experimental evidence was provided by radioligand binding with [^{3}H]candesartan as well as by measuring the effect of antagonist combinations on AT-mediated IP production in CHO-AT_1 cells. Furthermore, we compared the dissociation rate of these antagonists by these two experimental approaches.

MATERIALS AND METHODS

Materials

Candesartan [13, 22]), EXP3174 [23], losartan [23], and irbesartan [12] were obtained from AstraZeneca. AT was obtained from Sigma and *myo*[^{3}H]inositol (20 Ci/mmol) was from Pharmacia/Amersham/Biotech. [^{3}H]Candesartan (22 Ci/mmol) was provided by Takeda Chemical Industries, Ltd. All other chemicals were of the highest grade commercially available.

Cell Culture

Chinese hamster ovary cells stably expressing the human angiotensin II AT_1 receptor (CHO-AT_1 cells) were obtained as described by Vanderheyden *et al.* [4] and were cultured in 75-cm^2 flasks in DMEM supplemented with L-glutamine (2 mM), 2% of a stock solution containing 5000 IU/mL penicillin and 5000 μg/mL streptomycin (Life Technologies), 1% (v/v) of a solution of MEM containing non-essential amino acids, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (Life Technologies). The cells were grown in 5% CO_2 at 37° until confluent.

[^{3}H]Candesartan Binding

Cells were plated in 24-well plates and cultured until confluence. Before the experiment, the cells were washed three times with 500 μL per well of DMEM at room temperature. After removal of the medium, 400 μL DMEM was added and the plate was then left for 15 min at 37°. For saturation binding assays, cells were incubated with increasing concentrations of [^{3}H]candesartan (final free concentrations ranged between 0.08 and 15 nM) in a volume of 500 μL at 37° for 30 min in the absence or presence of unlabelled antagonists at the indicated final concentrations. For the [^{3}H]candesartan dissociation experiments, cells were incubated with 1.5 nM radioligand in final volume of 500 μL for 30 min at 37°. The dissociation of the radioligand was initiated by the addition of 1 μM candesartan in the absence or presence of 10 μM EXP3174, irbesartan, or losartan for the indicated periods of time. The dissociation rate constants of unlabelled antagonists were determined by measuring their ability to slow down the association rate of [^{3}H]candesartan by preincubation of the cells for 30 min at 37° with unlabelled candesartan (10 nM), EXP3174 (10 nM), irbesartan (100 nM), or losartan (10 μM). Cells were then briefly washed twice with cold PBS (containing 0.132 g/L $CaCl_2.2H_2O$, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 0.1 g/L $MgCl_2.6H_2O$, 8 g/L NaCl, and 1.44 g/L $Na_2HPO_4.2H_2O$) and incubated for the indicated periods of time with a final concentration of 1.5 nM [^{3}H]candesartan in DMEM. At the end of the experiments, the 24-well plates were placed on ice and the cells washed three times with PBS at 4°, pH 7.4. After the last aspiration of buffer, 500 μL of a 0.2 M sodium hydroxide solution was added to each well and the plates were further incubated for 1 hr at room temperature. Then, 400 μL of the solution was transferred into a scintillation vial, 3 mL of scintillation liquid (Optisafe from Wallac) was added, and the vials were counted for 3 min in a liquid scintillation counter. Non-specific binding, measured in the presence of 0.1 μM unlabelled candesartan, was subtracted from the total binding to yield specific [^{3}H]candesartan binding. The calculation of the binding parameters from the association binding curves (k_{obs}) and saturation binding curves (apparent K_d and B_{max} values) was performed by non-linear regression analysis using GraphPad Prism. Data points were the means ± SEM of three to four experiments with duplicate or triplicate determinations each.

The apparent dissociation rate constants ($k_{-1(I)}$) of the unlabelled antagonists were calculated by computer-assisted

iteration assuming a competitive interaction between [^{3}H]candesartan and the other antagonists. At the concentrations used in the present study, the unlabelled antagonists produced full inhibition of [^{3}H]candesartan binding [5]. Hence, after preincubation of the cells with antagonist (I), it was assumed that all the receptors (R) were occupied at the onset of the subsequent incubation with [^{3}H]candesartan (i.e. [R.I] = [R_{tot}] at t = 0). During this incubation, the dissociation of unlabelled antagonists was simulated according to a first-order reaction (i.e. d[R]/dt = $k_{-1(I)}$.[R.I]), and association of [^{3}H]candesartan (L*) was simulated according to a pseudo first-order reaction (i.e. d[R.L*]/dt = k_{obs}.[R].[L*]). Changes in [R] and [R.L*] were calculated at time intervals of 0.01 min for up to 4 hr, and $k_{-1(I)}$ values were adjusted until the computed [R.L*] versus time plots yielded the same apparent first-order rate constant as the experimental values.

IP Accumulation

The cells were plated in 24-well plates and cultured near to confluence. The medium was replaced by supplemented DMEM (see Cell Culture section above) containing 1 μCi/mL *myo*[^{3}H]inositol, and the cells were grown for a further 20 hr in 5% CO_2 at 37° until confluence. To investigate the effect of the antagonists on AT concentration–response curves, the cells were first washed twice with DMEM and left in 400 μL of DMEM containing 10 mM LiCl for 15 min at 37°. Preincubations were initiated by addition of 50 μL medium without (controls) or with antagonists and proceeded at 37° for 30 min. When cells were preincubated with two antagonists, both were added simultaneously. Subsequent incubations were initiated by adding 50 μL of medium alone (basal accumulation) or medium containing AT at various concentrations at 37° for 5 min. Similarly, antagonist inhibition curves were performed by preincubation of the cells with increasing concentrations of insurmountable antagonists in the absence or presence of losartan, after which the cells were further incubated with 10 μM AT at 37° for 5 min. The IC_{50} values of the inhibition curves with candesartan were calculated by non-linear regression analysis and used to calculate the pK_B values of losartan by Schild regression analysis according to Arunlakshana and Schild [24].

For the recovery experiments, the cells were first washed twice with DMEM (500 μL per well). After antagonist preincubation at 37° for 30 min, the cells were washed three times with 500 μL DMEM, left in DMEM containing 1 μM losartan for the indicated periods of time (i.e. washout time) at 37°, and washed three times with DMEM again. Subsequently, the incubations were started by adding 50 μL of medium without (basal accumulation) or with 0.1 μM AT (final concentration) and incubated at 37° for 5 min. The IP accumulation represented the measurement of mono-, bis-, and trisphosphates as described by Vanderheyden *et al.* [4]. Under these conditions, basal and AT (10 μM)-stimulated IP accumulation corresponded to 170 ± 55 and 1042 ± 299 cpm per well (mean ± SD). For the concentration–effect curves, the responses were given as percentages of the maximal AT response (E_{max}) in the absence of antagonist pretreatment. For the recovery experiments, responses were given as percentages of the matching control agonist stimulation without antagonist pretreatment. All values were means ± SEM of three to four experiments with triplicate determinations each.

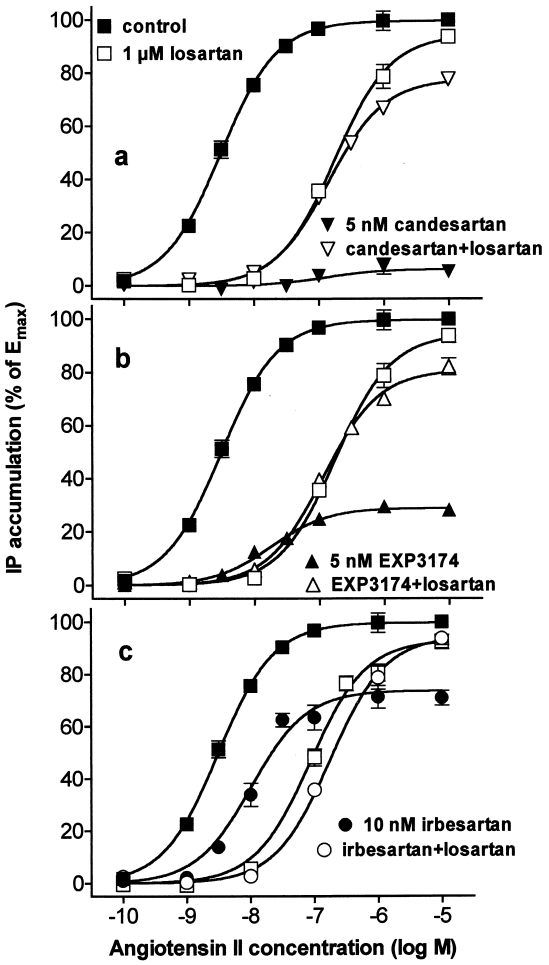

FIG. 1. Effect of antagonist (a: candesartan, b: EXP3174, c: irbesartan) preincubation in the absence or presence of 1 μM losartan, or of losartan alone, of CHO-AT_1 cells on the concentration-dependent IP production by AT. The values are the means ± SEM of 3 to 4 experiments. The corresponding pEC_{50} and E_{max} values are given in Table 1 and are given as percentages of the E_{max} without antagonist pretreatment (control).

RESULTS

Mutual Competitivity among Antagonists

Incubation of the CHO-AT_1 cells with AT for 5 min produced a concentration-dependent increase in IP accumulation with an EC_{50} of 3.1 nM (Fig. 1, Table 1). Preincubation of the cells for 30 min with the surmountable

TABLE 1. Effect of antagonist pretreatment on AT-induced IP accumulation in CHO-AT_1 cells

Condition	pEC_{50}	E_{max} (% of control)
Control	8.51 ± 0.01	100 ± 0.57
Losartan (1 μM)	6.76 ± 0.07*	94 ± 2.93
Candesartan (5 nM)	6.90 ± 0.40*	6.23 ± 1.20*
Candesartan (5 nM) + losartan (1 μM)	6.85 ± 0.03*	78 ± 1.11*
EXP3174 (5 nM)	7.75 ± 0.05*	29 ± 0.78*
EXP3174 (5 nM) + losartan (1 μM)	6.95 ± 0.04*	81 ± 1.69*
Irbesartan (10 nM)	8.00 ± 0.09*	74 ± 3.30*
Irbesartan (10 nM) + losartan (1 μM)	7.07 ± 0.07*	93 ± 3.09

The pEC_{50} and E_{max} values were calculated by non-linear regression analysis of the angiotensin II concentration–response curves (shown in Fig. 1) and were the averages ± SEM of at least three independent experiments.

*Statistically significant difference ($P < 0.05$) from control values, assessed by a non-paired Mann–Whitney test with two-tailed P values.

antagonist losartan (1 μM) caused a rightward shift of the concentration–response curve of AT without altering the maximal effect. Preincubation of the cells with the insurmountable antagonists candesartan (5 nM), EXP3174 (5 nM), or irbesartan (10 nM) produced an additional, substantial reduction in the maximal response to AT. This reduction was almost completely abolished for each of the insurmountable antagonists when the preincubation was performed in the simultaneous presence of 1 μM losartan (Fig. 1, Table 1). An alternative way to represent the antagonist data was to measure the AT (10 μM)-induced IP production after pretreatment of the cells with increasing concentrations of the insurmountable antagonist in the absence or presence of losartan. As shown in Figs. 2 and 3, the obtained concentration–inhibition curves of candesartan, EXP3174, and irbesartan were biphasic, with the most potent component corresponding to insurmountable inhibition and the least potent component to surmountable inhibition [6]. In the presence of losartan, the insurmountable inhibition curves were shifted to the right. This reversal was concentration-dependent for losartan, and the corresponding pK_B value was 7.7 (Fig. 2).

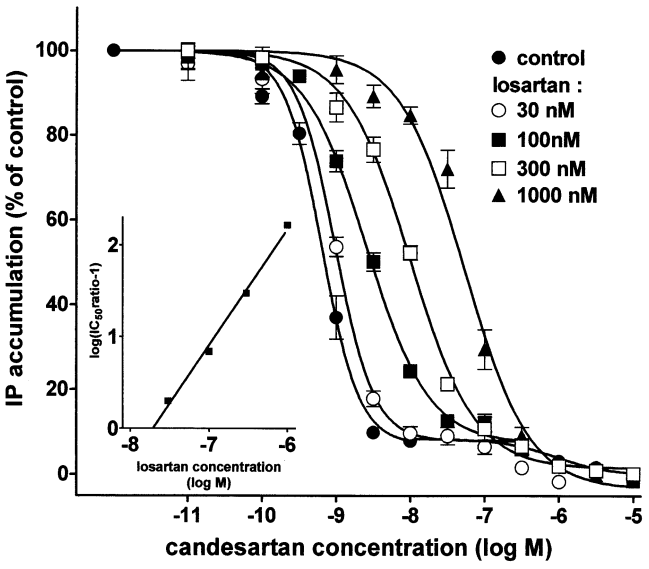

FIG. 2. Candesartan concentration–inhibition curve of AT (10 μM)-induced IP production in CHO-AT_1 cells in the absence or presence of the indicated concentrations of losartan. Values are the means ± SEM of 3 to 4 experiments as percentages of IP production without antagonist pretreatment. Inset: corresponding Schild regression of the reversal of candesartan inhibition by losartan.

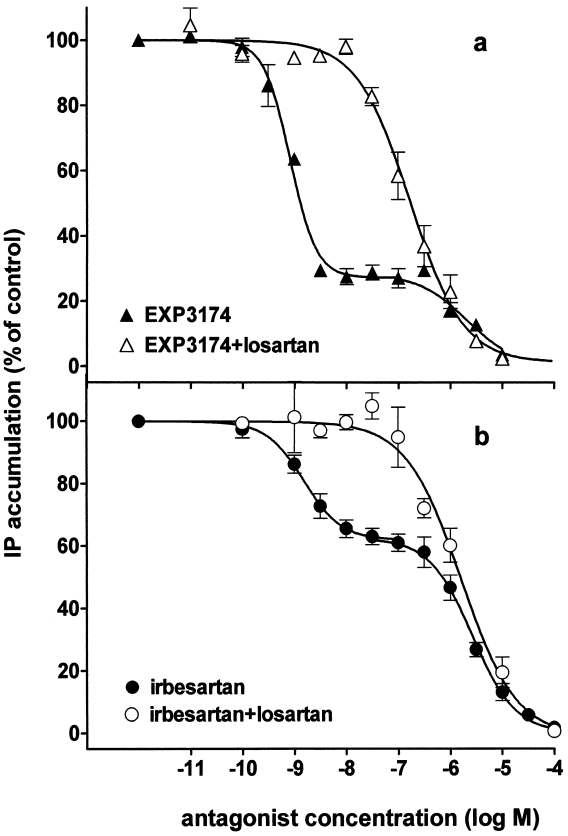

FIG. 3. Concentration–inhibition curve of AT (10 μM)-induced IP production in CHO-AT_1 cells by EXP3174 (a) and irbesartan (b) in the absence or presence of 1 μM losartan. Values are the means ± SEM of 3 to 4 experiments and are given as percentages of IP production without antagonist pretreatment.

TABLE 2. [^{3}H]Candesartan saturation binding parameters to CHO-AT_1 cells

Condition	Concentration (nM)	K_d (nM)	B_{max} (fmol/mg protein)
Control		0.13 ± 0.03	489 ± 34
Candesartan	0.5	0.29 ± 0.06*	481 ± 34
	1.5	0.64 ± 0.09*	490 ± 31
	5.0	4.46 ± 0.77*	370 ± 20
EXP3174	3	0.84 ± 0.22*	498 ± 46
	10	2.00 ± 0.36*	499 ± 35
Irbesartan	10	0.59 ± 0.12*	499 ± 38
	30	1.47 ± 0.20*	512 ± 40
Losartan	100	0.66 ± 0.186*	504 ± 42
	300	1.82 ± 0.40*	489 ± 38

The apparent K_d and B_{max} values were the averages ± SEM of four independent experiments (triplicate determinations) and were calculated by non-linear regression analysis using Graphpad Prism software. The free concentration of [^{3}H]candesartan ranged between 0.08 and 15 nM.

*Statistically significant difference ($P < 0.05$) from control values, assessed by a non-paired Mann–Whitney test with two-tailed P values.

Saturation binding of [^{3}H]candesartan to its receptors in CHO-AT_1 cells was measured either in the absence or presence of unlabelled candesartan, EXP3174, irbesartan, and losartan. As shown in Table 2, the unlabelled ligands did not substantially affect the maximal binding capacity of [^{3}H]candesartan in these co-incubation experiments. Instead, they produced a significant, dose-dependent increase in the apparent K_d values of the radioligand. Dissociation of [^{3}H]candesartan from CHO-AT_1 cells was initiated by isotopic dilution of pre-equilibrated [^{3}H]candesartan with 1 μM unlabelled candesartan. The binding decreased exponentially. As illustrated in Fig. 4, the dissociation rate was unaffected when the isotopic dilution was performed by adding 1 μM unlabelled candesartan in combination with 10 μM losartan, EXP3174, or irbesartan.

Dissociation Rate of Unlabelled Antagonists

Reversal of the antagonist inhibition was measured in washout experiments. For this purpose, cells were incubated for 30 min with antagonist, washed, and left in fresh medium for the desired periods of time before the (0.1 μM) AT-mediated response was measured (Fig. 5). The inhibition of candesartan was only reversed slowly. The reversal was consecutively faster for EXP3174 and irbesartan and very fast for losartan. In parallel, experiments were also performed with 1 μM losartan in the washout medium (Fig. 5). At this concentration, losartan was able to competitively hinder the interaction between the AT_1 receptor and the other antagonists (Figs. 1–3). Compared to the washout experiments with medium only, the recovery of the response in candesartan- and EXP3174-pretreated cells was accelerated in the presence of losartan. However, the recovery from irbesartan was not significantly changed. The order of the recovery rates for the investigated antagonists remained unchanged as well (Table 3). Kinetic experiments, in which CHO-AT_1 cells were incubated with 1.5 nM [^{3}H]candesartan for increasing periods of time, yielded a k_{obs} value of 0.15 ± 0.02 min^{-1} (Fig. 6). The association of [^{3}H]candesartan was delayed when the cells were preincubated with unlabelled antagonists and then washed twice. This delay was appreciable when the cells were pretreated with unlabelled candesartan (k_{obs} = 0.0043 ± 0.0010 min^{-1}), intermediate for EXP3174 (k_{obs} = 0.020 ± 0.001 min^{-1}), and minimal for irbesartan (k_{obs} = 0.033 ±

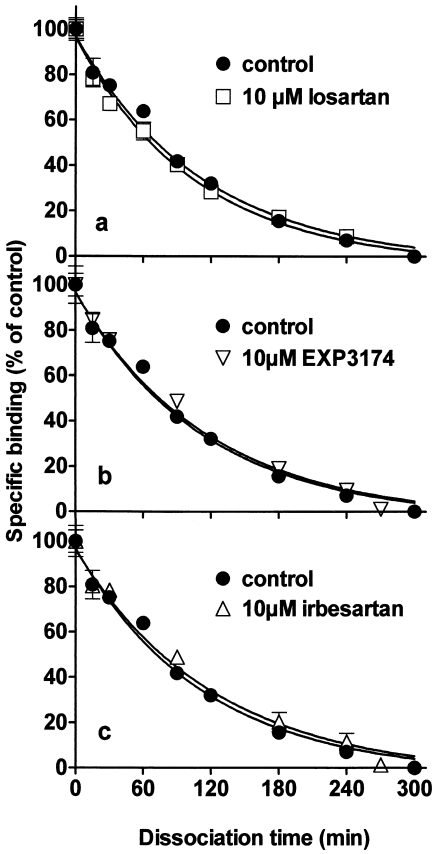

FIG. 4. Dissociation of [^{3}H]candesartan from CHO-AT_1 cells initiated by the addition of 1 μM candesartan in the absence (control) or presence of 10 μM losartan (a), 10 μM EXP3174 (b), or 10 μM irbesartan (c). Values (means ± SEM of 3 to 4 experiments) are given as percentages of the specific radioligand binding after 30 min of incubation at 37° without addition of unlabelled antagonist.

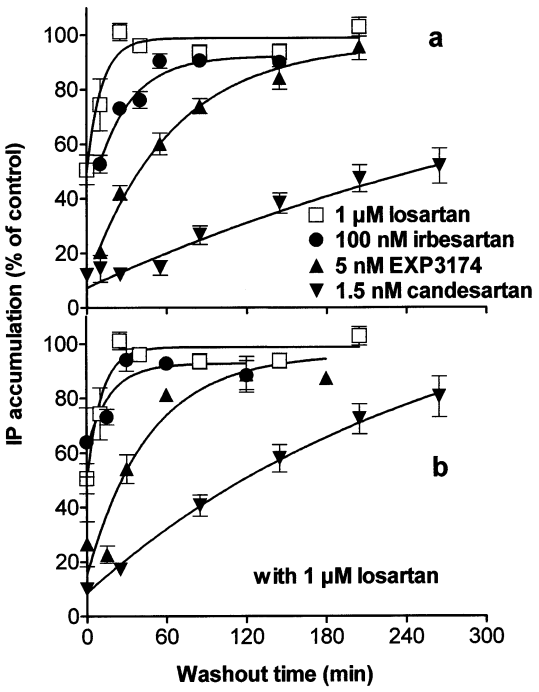

FIG. 5. Recovery of AT (0.1 μM)-induced IP production after antagonist preincubation of CHO-AT_1 cells and subsequent washout of the cells in medium without (a) or with 1 μM losartan (b) for the indicated periods of time. The values (means ± SEM of 3 to 4 experiments) are given as percentages of the matching control AT responses without antagonist pretreatment.

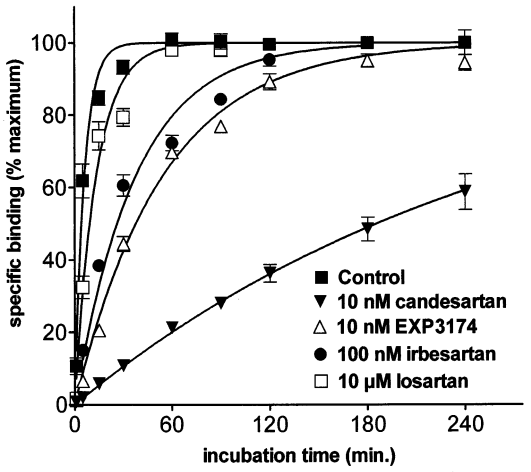

FIG. 6. Effect of antagonist preincubation of CHO-AT_1 cells on the association rate of [^{3}H]candesartan as described in the Methods section. Values (means ± SEM of 3 to 4 experiments) are given as percentages of specific binding after 60-min radioligand incubation without antagonist pretreatment (control).

0.002 min^{-1}) and losartan (k_{obs} = 0.082 ± 0.006 min^{-1}). Because of the competitive nature of the binding of [^{3}H]candesartan and the other antagonists to the AT_1 receptor (Table 2), this delay was considered to reflect the dissociation of the unlabelled ligand–receptor complexes. In addition, potential rebinding of dissociated unlabelled antagonists should be prevented by [^{3}H]candesartan itself, since its concentration (1.5 nM) was in excess of that of the receptors (≈0.1 nM) and as it is the most potent of the antagonists used [5]. Based on these considerations, the delayed association of [^{3}H]candesartan was utilized to calculate apparent first-order dissociation rate constants (k_{-1}) for the unlabelled antagonist–receptor complexes (Table 3).

DISCUSSION

We have investigated whether insurmountable and surmountable AT_1 receptor antagonists bind to a syntopic binding site in a competitive fashion or whether they interact with each other by an allosteric mechanism. Whilst there is recent evidence that both types of antagonists are competitive with AT [6, 25], it is proposed that an allosteric interaction between the AT_1 antagonist EXP3174 and AT may be responsible for its insurmountable behavior. Moreover, the recent three-

TABLE 3. Dissociation rate constants for AT_1 antagonists derived from [^{3}H]candesartan binding and functional recovery experiments in CHO-AT_1 cells

			IP recovery			
	[^{3}H]Candesartan association		Washout + losartan		Washout without losartan	
	k_{-1} (min^{-1})	$t_{1/2}$ (min)	k_{-1} (min^{-1})	$t_{1/2}$ (min)	k_{-1} (min^{-1})	$t_{1/2}$ (min)
Candesartan (1.5 nM)	0.0046 ± 0.0017	152 ± 58	0.0038 ± 0.0009	185 ± 46	0.0022 ± 0.0003	315 ± 43
EXP3174 (5 nM)	0.022 ± 0.004	31 ± 6	0.022 ± 0.007	33 ± 11	0.016 ± 0.003	44 ± 7
Irbesartan (100 nM)	0.041 ± 0.009	17 ± 4	0.057 ± 0.028	12 ± 6	0.038 ± 0.013	18 ± 6
Losartan (10 μM)	0.13 ± 0.03	5.2 ± 1.1	NM	NM	NM	NM

Dissociation rate constants (k_{-1}) and corresponding half-lives ($t_{1/2}$) were derived from: (a) slowing of the [^{3}H]candesartan association rate after pretreatment of the CHO-AT_1 cells with AT_1 antagonist, washing of the cells, and further incubation; and (b) recovery from insurmountable inhibition of AT (0.1 μM)-induced IP accumulation by AT_1 antagonists and washout of the cells with DMEM either without or containing 1 μM losartan. NM, not measurable.

dimensional receptor model of Inoue *et al.* [26] suggested that surmountable antagonists share the same binding sites with agonists, whereas insurmountable antagonists do not. On the other hand, a number of AT_1 receptor mutation studies [27, 28] indicate that both types of antagonists bind in distinct manners to a partially overlapping site rather deep between the membrane-spanning segments of the receptor, which suggests that they can displace each other in a competitive fashion. Binding studies were performed on intact CHO-AT_1 cells with the insurmountable antagonist [^{3}H]candesartan. Binding of this antagonist was shown to follow the law of mass action [5], which suggests the presence of a single class of binding sites. When analyzing the saturation binding curves of [^{3}H]candesartan, we found that the simultaneous presence of unlabelled candesartan, EXP3174, irbesartan, or losartan caused a dose-dependent increase in the corresponding apparent K_d values, whereas the B_{max} values were not altered significantly (Table 2). Such findings are generally interpreted in terms of a competitive interaction. Discriminative evidence also comes from kinetic experiments in which the dissociation of [^{3}H]candesartan from intact CHO-AT_1 cells was evaluated by the addition of an excess of unlabelled candesartan with or without EXP3174, irbesartan, or losartan. The rationale for this approach is based on the fact that a) the supramaximal concentration of candesartan prevents (re)association of [^{3}H]candesartan to the receptor sites and that b) if the addition of the other antagonists alters the dissociation rate of [^{3}H]candesartan, they must do so by interacting at a different allosteric site [29]. Yet, it is clearly shown in Fig. 4 that the dissocation rate of [^{3}H]candesartan in the presence of unlabelled candesartan and the other antagonists was the same as in the presence of candesartan alone. Hence, these isotope dilution experiments, as well, do not plead in favor of allosteric interactions between antagonists and the AT_1 receptor.

Functional experiments involved the measurement of AT-mediated IP production in cells pretreated with the insurmountable antagonists candesartan, EXP3174, and irbesartan. These antagonists decreased the maximal response to various degrees, this effect being abolished when the surmountable antagonist losartan was present simultaneously. The restoration of the maximal AT effect by losartan has also been reported for the rabbit aortic strip contraction model [9, 10, 14, 17, 20] and for the pithed rat diastolic blood pressure model [30]. This restoration is generally regarded to reflect the competitive, syntopic action of surmountable and insurmountable antagonists and, accordingly, was already demonstrated by Wienen *et al.* [25] and Wong and Timmermans [30] to be concentration-dependent. To gain further insight into this issue, we have adapted the approach described by Frenken *et al.* [31], in which the concentration–inhibition of an insurmountable 5-HT antagonist was performed in the presence of a surmountable antagonist. This approach consisted of the measurement of the inhibition curves of candesartan, EXP3174, and irbesartan, in the absence or presence of losartan. The concentration of AT (10 μM) was maximally effective, and under these conditions the resulting inhibition curves of the insurmountable antagonists were previously shown to become biphasic with the most potent component corresponding to insurmountable inhibition and the least potent to surmountable inhibition [6]. Whereas the insurmountable component of candesartan inhibition represented 95% of the control response, it was 70% for EXP3174 and 30% for irbesartan. As shown in Fig. 2, the insurmountable inhibition by candesartan underwent a concentration-dependent rightward shift by losartan. The corresponding pK_B values for losartan calculated by Schild regression analysis (7.7) are similar to those obtained from losartan inhibition of AT-induced aortic strip contraction (8.2–8.5 [8, 10, 32]) as well as IP production in preincubated CHO-AT_1 cells (7.9 [4]). Similarly, the insurmountable components of EXP3174 and irbesartan concentration–inhibition underwent a rightward shift in the presence of 1 μM losartan (Fig. 3).

It has been shown that addition of unlabelled antagonists accelerates the dissociation of [^{3}H]candesartan from CHO-AT_1 cells when compared to washing the cells with fresh medium [5]. The proposal that this effect reflects the ability of unlabelled antagonists to prevent rebinding [33] rather than an allosteric modulation is supported by the results in the present manuscript. This prompted us to explore different approaches to obtain a correct estimation of the dissociation rate of insurmountable AT_1 antagonists, i.e. in the absence of potential rebinding. In the first approach, we measured the recovery of AT responses after antagonist pretreatment of the CHO-AT_1 cells. When such recovery experiments were performed previously by replacement of the antagonist-containing medium with fresh medium, rebinding of released antagonist could occur [33]. This would result in an underestimation of the true dissociation rate of insurmountable antagonists. This phenomenon is indeed observed in our experimental set-up (Fig. 5 and Table 3), since the recovery from candesartan and EXP3174 was accelerated when the cells were washed with medium that contained 1 μM losartan. On the other hand, nearly no appreciable acceleration of the recovery was observed for irbesartan.

In the second approach, we have compared the slowing of the association rate of [^{3}H]candesartan after pretreatment of CHO-AT_1 cells with unlabelled antagonists, a method similar to that described by Hara *et al.* [15] and one which relies on a competitive interaction between these ligands. In these experiments, [^{3}H]candesartan not only serves as the radioligand but also prevents the (re)occupation of the receptor by the unlabelled antagonists. Thus, in the absence of rebinding, these two experimental approaches provided similar antagonist dissociation kinetics (Table 3). Whereas the half-life of the unlabelled candesartan–receptor complex was approximately 150 min, the dissociation was appreciably faster for EXP3174. The dis-

sociation was still faster for irbesartan, in agreement with the earlier observation that there is already a noticeable restoration of the response in irbesartan-pretreated CHO-AT_1 cells during the subsequent 5-min challenge with AT [6]. The already very fast dissociation of irbesartan in the absence of losartan also precluded a clear-cut assessment of its potential rebinding. Finally, and in agreement with its surmountable nature in the functional experiments, the dissociation was extremely fast for losartan. We have no evidence that the insurmountable antagonists EXP3174 and irbesartan affect the desensitization of AT-induced IP accumulation, since the responses returned to values comparable to the corresponding control values (Fig. 5). Whether this is also the case for candesartan is not clear due to its extreme slow dissociation. Furthermore, all the investigated antagonists do not affect basal IP accumulation (data not shown).

In conclusion, [^{3}H]candesartan saturation binding to intact CHO-AT_1 and functional data suggest that the AT_1 antagonists candesartan, EXP3174, irbesartan, and losartan bind to a syntopic binding site in a competitive fashion. Based on this property, two different experimental approaches allowed us to estimate the dissociation rate of these AT_1 antagonists in the absence of rebinding.

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