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A two-state receptor model for the interaction between angiotensin II type 1 receptors and non-peptide antagonists

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Abstract

The interaction between non-peptide antagonists and the human angiotensin II type 1 (AT_1) receptor in CHO-K1 cells was investigated by incubating the cells with antagonist, followed by a brief exposure to angiotensin II and measurement of the resulting inositol phosphate accumulation. The experimental data, expressed either as angiotensin II concentration–response curves or as antagonist concentration–inhibition curves, were in good agreement with computer-generated data according to a single-state model for the surmountable antagonist losartan and according to a two-step, two-state receptor model for the insurmountable antagonists candesartan, EXP3174, and irbesartan. Experimental and computer-generated data concerning the simultaneous exposure of the receptors to EXP3174 and losartan indicated that losartan produced a concentration-dependent restoration of the maximal response (angiotensin II concentration–response curves) as well as a rightward shift of the insurmountable portion of the EXP3174 inhibition curves, thus counteracting the higher-affinity EXP3174 binding. In conclusion, these findings provide further support for the concept that insurmountable and surmountable AT_1 antagonists are mutually competitive and that insurmountable antagonist–receptor complexes may adopt different states.

Keywords: AT_1 antagonists; CHO cells; Inositol phosphate; Candesartan; Irbesartan; EXP3174; Losartan; Surmountable; Insurmountable

1. Introduction

Several non-peptide AT_1 receptor antagonists have been developed for the clinical treatment of hypertension and congestive heart failure [1]. The interaction between these antagonists and the AT_1 receptor has often been studied by *in vitro* contraction experiments on rabbit aorta rings/strips and rat portal vein. More recently, relevant information was also obtained by measuring angiotensin II-induced IP production in cell lines expressing transfected AT_1 receptors [2–5]. Both kinds of studies plead for the existence of two categories of antagonists. Surmountable antagonists, such as losartan, produce parallel rightward shifts of the angiotensin II concentration–response curve and do not affect the maximal response [3,6]. In contrast, insurmountable antagonists decrease the maximal response to angiotensin II. This decrease may be partial for antagonists such as irbesartan and EXP3174 (the active metabolite of losartan) to almost complete for antagonists such as candesartan [3,7–10].

Several theories have been advanced over the past ten years to explain the molecular mechanism of surmountable and insurmountable AT_1 receptor antagonists. They include the presence of allosteric binding sites on the receptor [11], slowly interconverting receptor conformations [12–14], slow removal of the antagonist from tissue compartments, cells, or matrix surrounding the receptor [15,16], coexistence of different receptor subpopulations [17], and the ability of the antagonist to modulate the amount of internalized receptors [7] or slow dissociation of the antagonist–receptor

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Abbreviations: AT, angiotensin II; 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 essential 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 triphosphates; irbesartan, 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl) methyl]2-imidazolin-5-one); and losartan, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole.

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complex [18–22]. This latter possibility has been favored by most authors and is now also strongly supported by the observation that insurmountable AT_1 receptor antagonists only depress the maximal response to angiotensin II when they are pre-exposed to the receptors. Interestingly, the same antagonists are able to produce rightward shifts of the angiotensin II concentration–effect curve without depressing the maximal response when they are added simultaneously [5,23]. Similarly, antagonists which decreased the maximal binding capacity of labeled angiotensin II when added to the receptors first did not do so under co-incubation conditions [5]. These findings support the proposal that insurmountable AT_1 receptor antagonists inhibit the response to angiotensin II in a competitive fashion and that, when they are pre-exposed to the receptor, their antagonistic action may be so slowly reversible that it cannot be overcome during the ensuing exposure of the receptors to the agonist [5]. Surmountable antagonists are competitive with angiotensin II as well, but their action is readily reversible so that it can be completely overcome by the subsequently added agonist.

It is intriguing why most of the insurmountable antagonists only produce a partial decrease of the maximal response to angiotensin II. As a possible explanation for this phenomenon, it was recently proposed that antagonist–AT_1 receptor complexes may adopt a fast reversible (surmountable) as well as a tight-binding (insurmountable) state and that there is an equilibrium between both states that is dependent on the chemical nature of the bound antagonist [5].

In the present study, we correlated experimental data describing the interaction between insurmountable (candesartan, EXP3174, and irbesartan) AT_1 receptor antagonists and their receptors with computer-generated data according to a two-step, two-state receptor model. Experimental and computer-generated data concerning the simultaneous exposure of the receptors to surmountable and insurmountable AT_1 antagonists also provided further support for the concept that these antagonists are mutually competitive.

2. Materials and Methods

2.1. Materials

Candesartan [9], EXP3174 [13], losartan [13], and irbesartan [8] were obtained from AstraZeneca. Angiotensin II was obtained from Sigma, *myo*-[^{3}H]inositol (20 Ci/mmol) was from Pharmacia/Amersham/Biotech. All other chemicals were of the highest grade commercially available.

2.2. Cell culture

CHO-K1 cells stably expressing the human angiotensin II AT_1 receptor (CHO-AT_1 cells) were obtained as described by Vanderheyden *et al*. [3] and were cultured in 75-cm^2 flasks in DMEM which was supplemented with L-glutamine (2 mM), 2% of a stock solution containing 5000 IU/mL of penicillin and 5 μg/L of 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.

2.3. IP accumulation

The cells were plated in 24-well plates and cultured until confluent. The medium was replaced by supplemented DMEM (see preceding section) containing 1 μCi/mL of *myo*-[^{3}H]inositol, and the cells were incubated for 20 hr in 5% CO_2 at 37° and finally washed twice with DMEM (500 μL per well). To investigate the effect of the antagonists on angiotensin II-mediated responses, the cells were first washed twice with DMEM and then 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 at the indicated final concentrations and continued at 37° for the indicated periods of time or for 30 min (for inhibition curves and dose–response curves). When the cells were preincubated with two antagonists, both were added simultaneously. Subsequent incubations (at 37° for 5 min) were initiated by adding 50 μL of medium alone (basal accumulation) or medium containing angiotensin II at various concentrations. The IP accumulation represented the measurement of mono-, bis-, and trisphosphates and was determined as described by Vanderheyden *et al*. [3]. For the antagonist concentration–inhibition curves, the responses were given as percentage of the maximal angiotensin II response without antagonist pretreatment. All values were means ± standard error of the mean of three to four experiments with triplicate determinations for each.

2.4. Computer-assisted simulations

Antagonist and angiotensin II–receptor interactions were simulated according to the following reaction mechanisms:

$$A + R \underset{k_{-1a}}{\overset{k_{1a}}{\rightleftharpoons}} A.R$$

A reversible bimolecular reaction represents the angiotensin II (A)–receptor (R) interaction, and the response is at all times proportional to the concentration of the agonist–receptor (A.R) complex.

$$S + R \underset{k_{-1s}}{\overset{k_{1s}}{\rightleftharpoons}} S.R$$

A reversible bimolecular reaction also represents the surmountable antagonist (S)–receptor interaction,

$$I + R \underset{k_{-1i}}{\overset{k_{1i}}{\rightleftharpoons}} I.R \underset{k_{-2i}}{\overset{k_{2i}}{\rightleftharpoons}} I.R^*$$

Table 1
Parameters for the computer-assisted simulation of the curves in Figs. 2–4

Equations	$S + R \underset{k_{-1s}}{\overset{k_{1s}}{\rightleftharpoons}} S.R$		$I + R \underset{k_{-1i}}{\overset{k_{1i}}{\rightleftharpoons}} I.R \underset{k_{-2i}}{\overset{k_{2i}}{\rightleftharpoons}} I.R^a$			
Equilibrium dissociation constants: (unit)	K_{ds} (nM)	K_{d1i} (nM)			K_{d2i} None	macroscopic K_{di}* (nM)
kinetic constants/ratios (unit)	k_{-1s}/k_{1s}	k_{-1i}/k_{1i}	k_{2i} ($M^{-1}.min^{-1}$)	k_{-2i} (min^{-1})	k_{-2i}/k_{2i}	
Losartan	7.5					
Irbesartan		1.5	0.077	0.104	1.35	0.86
EXP3174		2.2	0.084	0.022	0.26	0.45
Candesartan		2.5	0.121	0.0061	0.05	0.12

Kinetic parameters or ratios and derived equilibrium dissociation constants for the computer-assisted simulation of the curves shown in Figs. 2 to 4 for the antagonist–AT_1 receptor interaction in CHO-AT_1 cells determined as described in the Methods section.

[a] macroscopic $K_{di} = K_{d1i}.K_{d2i}/(K_{d2i} + 1)$.

for the insurmountable antagonist (I)–receptor interaction; R is the fast reversible (surmountable) state of the receptor and R* the tight-binding (insurmountable) state.

The ratio of the kinetic constants for association (k_{1s}, k_{1i}) and dissociation (k_{-1s}, k_{-1i}) of the antagonists and kinetic constants for the conversion of the antagonist–receptor complexes (k_{2i} and k_{-2i}) are given in Table 1. The kinetic constants for association (k_{1a}) and dissociation (k_{-1a}) of angiotensin II are arbitrarily set to 0.5 10^9 $M^{-1}.min^{-1}$ and 1.5 min^{-1} to yield an equilibrium dissociation constant (K_{da} = 3 nM) that corresponds to the experimental EC_{50} for angiotensin II-mediated IP production in CHO-AT_1 cells [3].

(i) Preincubation with antagonist. At time t = 0, [R] = 1, [I.R] (or [S.R]) = 0, [I.R*] = 0. The total receptor concentration is taken as unity. For preincubations with a single antagonist, subsequent variations in [R], [I.R], [S.R], and [I.R*] are calculated over very small time periods (d(t) = up to 1.2 msec) as follows:

$$d[R] = d(t).(k_{-1s}.[S.R] - k_{1s}.[S].[R]) \text{ or}$$

$$d[R] = d(t).(k_{-1i}.[I.R] - k_{1i}.[I].[R]) \quad (1)$$

$$d[S.R] = d(t).(k_{1s}.[S].[R] - k_{-1s}.[S.R]) \quad (2)$$

$$d[I.R] = d(t).(k_{1i}.[I].[R] - k_{-1i}.[I.R] + k_{-2i}.[I.R^*] - k_{2i}.[I.R]) \quad (3)$$

$$d[I.R^*] = d(t).(k_{2i}.[I.R] - k_{-2i}.[I.R^*]) \quad (4)$$

These variations are cumulated until the desired preincubation time (usually 30 min) is reached. Preincubations with surmountable and insurmountable antagonists are analyzed as above with d[S.R], d[I.R], and d[I.R*] corresponding to equations 2 to 4 and

$$d[R] = d(t).(k_{-1s}.[S.R] - k_{1s}.[S].[R] + k_{-1i}.[I.R] - k_{1i}.[I].[R]) \quad (5)$$

(ii) Incubation with antagonist and agonist. At time t = 0, [A.R] = 0 and values for [I.R], [S.R], and [I.R*] correspond to those calculated for the end of the preincubation. For preincubations with a single antagonist, subsequent incubations with agonist and antagonist are analyzed as above with d[I.R], d[S.R], and d[I.R*] corresponding to equations 2 to 4 and

$$d[R] = d(t).(k_{-1s}.[S.R] - k_{1s}.[S].[R] + k_{-1a}.[A.R] - k_{1a}.[A].[R]) \text{ or}$$

$$d[R] = d(t).(k_{-1i}.[I.R] - k_{1i}.[I].[R] + k_{-1a}.[A.R] - k_{1a}.[A].[R]) \quad (6)$$

$$d[A.R] = d(t).(k_{1a}.[A].[R] - k_{-1a}.[A.R]) \quad (7)$$

For preincubations with a mixture of surmountable and insurmountable antagonists, subsequent incubations with agonist and antagonists are analyzed as above with d[I.R], d[S.R], d[I.R*], and d[A.R] corresponding to equations 2, 3, 4, 7, and

$$d[R] = d(t).(k_{-1s}.[S.R] - k_{1s}.[S].[R] + k_{-1i}.[I.R] - k_{1i}.[I].[R] + k_{-1a}.[A.R] - k_{1a}.[A].[R]) \quad (8)$$

When the responsiveness of the receptors is constant over time, the response (i.e. accumulation of IP produced with time) is related to the maximal response (i.e. constant stimulation of all the receptors) according to:

$$\frac{\sum_{t=0}^{x} [A.R]_t}{x}$$

with x corresponding to total incubation time with agonist/d(t).

2.5. Appropriate parameters for the computer model

Insurmountable antagonists yielded biphasic inhibition curves. IC_{50} values for the most potent/insurmountable (IC_{50i}) and least potent/surmountable (IC_{50s}) components and

the plateau values (corresponding to the proportion of surmountable inhibition) of the experimental and simulated inhibition curves were calculated by non-linear regression analysis (using GraphPad Prism). Parameters in the computer-assisted simulations were obtained on the basis of the following three considerations.

First, values for k_{-2i} were set to correspond to the dissociation rates of the insurmountable antagonist–receptor complexes as derived previously from [^{3}H]candesartan and [^{3}H]irbesartan binding and from the recovery of angiotensin II-induced IP production in EXP3174-preincubated CHO-AT_1 cells [4,24,25].

Second, to determine the other parameters (k_{2i} values and the k_{-1i},/k_{1i} ratios) for the two-state, two-step model, we investigated the consequences of varying their values on IC_{50} values and plateau values by non-linear regression analysis of computer-generated data. This procedure was adopted for all insurmountable antagonists. As illustrated for EXP3174, a hyperbolic relationship was found between the extent of insurmountable inhibition in the simulated inhibition curves and the k_{2i} values (Fig. 1A). A linear representation thereof could be obtained by comparing the reciprocal values (Fig. 1A, inset). Since this relation is only marginally dependent on k_{-1i}, and k_{1i}, it can be used to obtain relevant k_{2i} values by interpolation (i.e. k_{2i} corresponding to the experimentally determined extent of insurmountable inhibition).

Finally, it was also found that IC_{50i} and IC_{50s} (illustrated for EXP3174) depend on k_{2i} and that, when the latter parameter is kept constant, there is a linear relationship between log(IC_{50}) and log(k_{-1i}/k_{1i}) (shown for log[IC_{50i}] in Fig. 1B). Within the tested range, this linear relationship emerged irrespectively of the individual k_{-1i} and k_{1i} values (Fig. 1B). Hence, when k_{-2i} and k_{2i} are set as explained in the first two considerations above, this linear relationship can be used to calculate the k_{-1i}/k_{1i} ratio by interpolation. Since the IC_{50} values only depend on the k_{-1i}/k_{1i} ratio, the separate k_{-1i} and k_{1i} values could not be determined independently. Therefore, k_{1i} was arbitrarily set at 3.10^9 $M^{-1}.min^{-1}$ for the simulations.

The inhibition curve of the surmountable antagonist losartan was monophasic. In compliance with the other antagonists, k_{1s} was arbitrarily set at 3.10^9 $M^{-1}.min^{-1}$ and k_{-1s} was set to 22.5 min^{-1} to fit with the experimental curve.

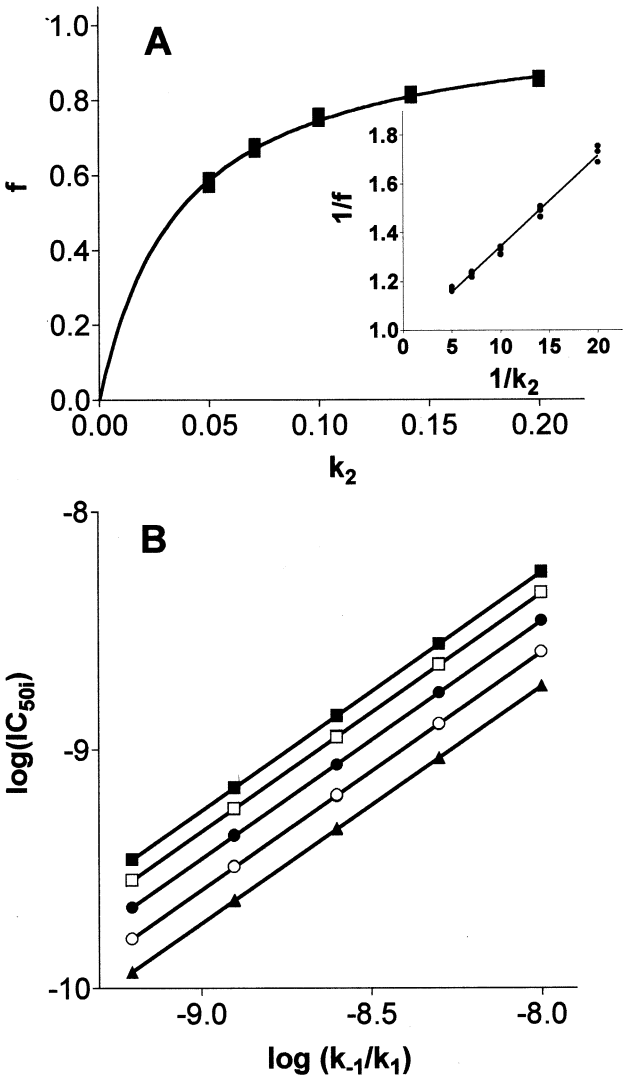

Fig. 1. (A) Relationship between the fraction of insurmountable inhibition by EXP3174 (represented as f) and k_2. The f values were calculated by non-linear regression analysis of computer-generated data points of an EXP3174 concentration–inhibition curve (angiotensin II concentration 10 μM) with varying k_1 ($1.5\ 10^9$, $3\ 10^9$, and $6\ 10^9$ $M^{-1}.min^{-1}$) and k_{-1} values (3.75, 7.5, and 15 min^{-1}) as outlined in the Methods section. Inset: Linear representation of the above relationship by plotting 1/f versus $1/k_2$. (B) Relationship between log(IC_{50i}) of the simulated EXP3174 concentration–inhibition curve and log(k_{-1}/k_1) values, when the k_2 values were set to 0.05 (■), 0.07 (□), 0.10 (●), 0.14 (○), or 0.2 $M^{-1}.min^{-1}$ (▲).

3. Results

CHO-AT_1 cells were preincubated for 30 min with increasing concentrations of antagonist, followed by a 5-min challenge with a maximally effective concentration of angiotensin II (10 μM). The resulting experimental IP accumulation data yielded a monophasic inhibition curve for losartan and biphasic curves for the insurmountable antagonists with plateau values at about 5% for candesartan, 25% for EXP3174, and 61% for irbesartan (Fig. 2, Table 2). The complexity of the experiments (involving a preincubation and an incubation step as well as potential hemi-equilibria) prompted us to simulate data based on differential equations (see Methods section) instead of a global function. Whereas a single-site model adequately describes the monophasic inhibition curve of losartan, a more complex description such as the proposed two-step, two-state model is required to explain the biphasic curves of the insurmountable antagonists candesartan, EXP3174, and irbesartan.

Parameters in these simulations (given in Table 1) were found as follows. Values for k_{-2i} were set to correspond to

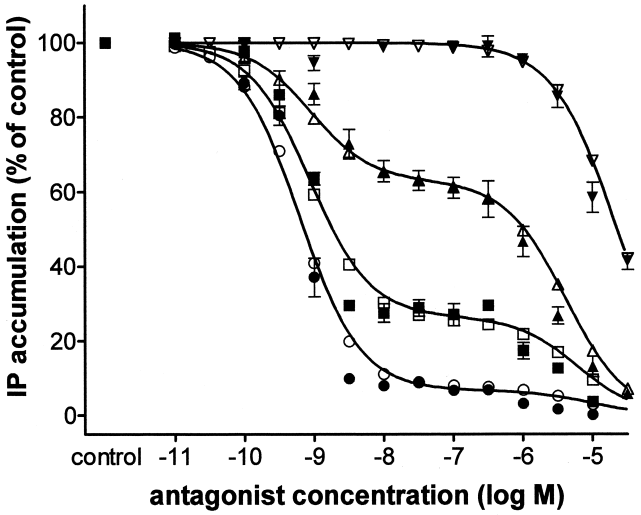

Fig. 2. Concentration–inhibition curve of candesartan (●), EXP3174 (■), irbesartan (▲), and losartan (▼) on the angiotensin II-mediated IP accumulation in CHO-AT_1 cells. Cells were preincubated with increasing concentrations of the antagonists (abscissa) for 30 min at 37°, after which 10 μM angiotensin II was added and the incubation continued for 5 min. Data points (average ± SEM of three independent experiments) are expressed as % of the angiotensin II response in the absence of antagonist. Curves were obtained by non-linear regression analysis of the computer-generated data for candesartan (○), EXP3174 (□), irbesartan (△), and losartan (▽) as described in the Methods section.

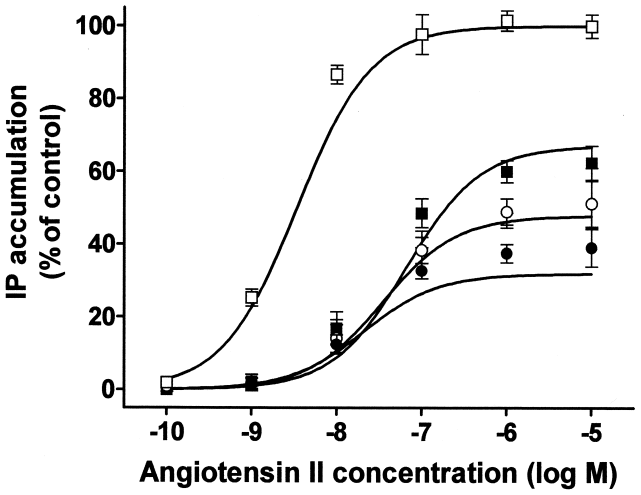

Fig. 3. Concentration–response curves of angiotensin II-mediated IP accumulation after CHO-AT_1 cells were preincubated for 30 min with 10 nM EXP3174 either alone (●) or in the presence of 30 (○) or 100 (■) nM losartan. Data are the average ± SEM of three independent experiments and are expressed as % of the maximal angiotensin II response after preincubation with medium alone (□). Curves were obtained by the non-linear regression analysis of computer-generated data described in Methods.

the dissociation rates of the insurmountable antagonist–receptor complexes that were previously estimated either directly (dissociation of [^{3}H]candesartan and [^{3}H]irbesartan) [4,24] or, for EXP3174, by indirect techniques (restoration of angiotensin II-mediated responses and delay of [^{3}H]candesartan binding in EXP3174-pretreated CHO-AT_1 cells) [25]. The other parameters were determined based on the relationship between the extent of insurmountable inhibition and k_{2i} and on the dependence of the IC_{50} values on the k_{-1}/k_1 ratio (Fig. 1). For losartan, this latter ratio is very close to the K_d-values previously obtained by competition binding studies with [^{3}H]angiotensin II and [^{3}H]candesartan [3,4]. Experimental and computer-generated antagonist inhibition data are compared in Fig. 2. The non-linear regression analysis of both types of data (Table 2) yielded curves with very similar IC_{50} values and, for insurmountable antagonists, similar plateau values.

Preincubation of the CHO-AT_1 cells for 30 min with 10 nM EXP3174 produced a rightward shift of the dose–response curve as well as a decline in the maximal response of subsequently added angiotensin (experimental data points in Fig. 3). The curves shown in this figure were computer-generated according to the parameters given in Table 2. When losartan was also included in the preincubation, both the experimental data and the curves revealed that losartan produced a dose-dependent rise in the maximal response to angiotensin II (Fig. 3). When cells were preincubated for 30 min with increasing concentrations of EXP3174 and subsequently with 10 μM angiotensin II, the simultaneous pres-

Table 2
Parameters calculated by non-linear regression analysis of computer-simulated and experimental antagonist inhibition curves

Antagonist	Data generation	$-\log(IC_{50i})$	$-\log(IC_{50s})$	f
Candesartan	Experimental	9.17 (9.00–9.34)	5.53 (4.79–6.26)	0.95 (0.89–0.99)
Candesartan	Simulated	9.21 (9.17–9.24)	5.02 (4.48–5.57)	0.93 (0.92–0.95)
EXP3174	Experimental	9.06 (8.82–9.30)	5.45 (4.68–6.21)	0.75 (0.67–0.84)
EXP3174	Simulated	9.06 (9.04–9.08)	5.22 (5.17–5.26)	0.74 (0.73–0.74)
Irbesartan	Experimental	8.86 (8.74–9.00)	5.58 (5.52–5.64)	0.37 (0.35–0.39)
Irbesartan	Simulated	9.04 (9.04–9.05)	5.39 (5.39–5.40)	0.37 (0.36–0.38)
Losartan	Experimental	NA	4.72 (4.62–4.82)	NA
Losartan	Simulated	NA	4.65 (4.63–4.66)	NA

IC_{50i} ($-$log M), IC_{50s} ($-$log M), and f (i.e. fraction of insurmountable inhibition) for the indicated antagonists calculated by non-linear regression analysis of computer-generated data points (simulated) and of experimental antagonist concentration–inhibition curves (experimental), both at a angiotensin II concentration of 10 μM. Experimental data points are the means of three to four separate experiments. The values between parentheses are the 95% confidence intervals of the non-linear regression analysis. NA, means not applicable.

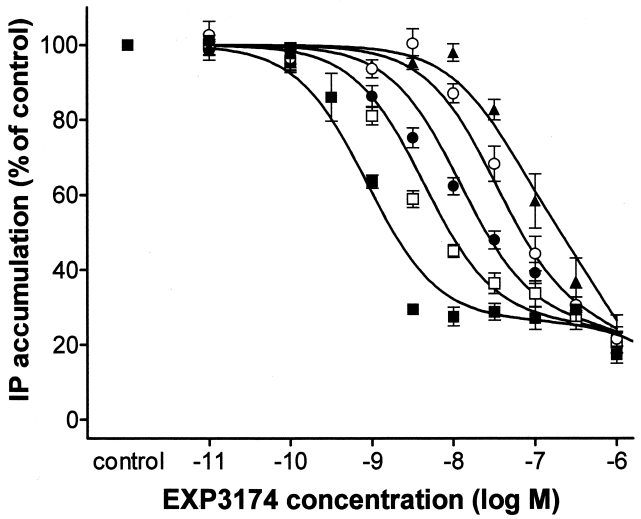

Fig. 4. Concentration–inhibition curve of EXP3174 on the angiotensin II-mediated IP accumulation in CHO-AT_1 cells: effect of losartan. Cells were preincubated for 30 min at 37° with increasing concentrations of EXP3174 either alone (■) or in the presence of 30 (□), 100 (●), 300 (○), or 1000 (▲) nM losartan, after which 10 μM angiotensin II was added and the incubation continued for 5 min. Data points (expressed as % of the angiotensin II response in the absence of antagonist) are the average ± SEM of three independent experiments, and curves were obtained by the non-linear regression analysis of computer-generated data described in Methods.

ence of losartan in the preincubation phase resulted in a dose-dependent rightward shift of the insurmountable portion of the inhibition curve (Fig. 4). This shift was comparable for the experimental data and for the computer-generated curves.

4. Discussion

AT_1 receptor antagonists only depress the maximal angiotensin II-mediated response when they are allowed to interact with the receptor before the agonist [5,23]. This depression, or insurmountable antagonism, is traditionally reported by presenting the effect of antagonists on angiotensin II concentration–response curves. It emerges from many reports that when the concentration of an insurmountable antagonist is gradually increased, it will depress the maximal response until a certain level is reached. For most of the insurmountable antagonists, this depression is only partial and its level varies from one molecule to another. However, when their concentration is further increased, all antagonists are still able to produce rightward shifts of the depressed concentration–response curve.

These phenomena can be better quantified when portraying the experimental data in the form of inhibition curves [5]. Such a representation is shown in Fig. 2 for the effect of the surmountable antagonist losartan and the insurmountable antagonists candesartan, EXP3174, and irbesartan on angiotensin II-mediated IP production in CHO-AT_1 cells. For each inhibition curve, the receptors are pre-exposed to a wide range of antagonist concentrations and the response is plotted for a single concentration of angiotensin II. When the angiotensin II concentration is sufficiently high, the inhibition curves have previously been shown to become biphasic [5]. The most potent component corresponds to insurmountable inhibition, since it is independent of the angiotensin II concentration. The least potent component corresponds to surmountable inhibition, since its IC_{50} is increased when the angiotensin II concentration is raised. The presence of a distinct plateau between these components facilitates the determination of the maximal extent of insurmountable inhibition by each antagonist. As these percentages are not affected by the preincubation time (between 10 and 120 min), it was proposed that insurmountable antagonist (I)–receptor (R) complexes may adopt a fast reversible (I.R) as well as a tight-binding (I.R*) state and that there is an equilibrium between these states that is dependent on the chemical nature of the bound antagonist.

The simplest model to describe the relationship between these states is a two-step mechanism in which the initial binding of the antagonist is fast and reversible and the bound antagonist then incites the receptor to adopt a tight-binding state:

$$\mathrm{I} + \mathrm{R} \underset{k_{-1i}}{\overset{k_{1i}}{\rightleftharpoons}} \mathrm{I.R} \underset{k_{-2i}}{\overset{k_{2i}}{\rightleftharpoons}} \mathrm{I.R^*}$$

When the k_{-2i} values were set to the experimentally determined dissociation rate, the computer-generated inhibition curves could be made to fit closely with the data from candesartan, EXP3174, and irbesartan concentration–IP inhibition experiments (Fig. 2, Table 1). Computer-assisted iterations as shown in Fig. 1 revealed that the maximal extent of insurmountable inhibition by each antagonist is mainly governed by the equilibrium between the surmountable and the insurmountable state of the receptor. Moreover the IC_{50} values of the surmountable and insurmountable components of the antagonist inhibition curves depend on this equilibrium as well as on k_{-1i}/k_{1i}. Interestingly, whereas k_{-2i} is quite variable between the different antagonists, the k_{-1i}/k_{1i} ratio and k_{2i} remain very similar for each of them.

Two-site curve fitting of the computer-generated and the experimental data yielded similar binding parameters (Table 2). This is in agreement with the concept that the antagonist–receptor complex may adopt a fast reversible and a tight-binding state, and a two-step mechanism to describe the relationship between these states. Two major deductions can be made when comparing the kinetic constants of each antagonist. First, it was reported in an earlier study [3] that the insurmountable nature of AT_1 receptor antagonists in functional studies is related to their long-lasting inhibition. Since k_{2i} values are comparable for candesartan, EXP3174, and irbesartan, the present model is in agreement with this conclusion, as it stipulates that the maximal extent of insur-

mountable inhibition by these antagonists is only dependent on their k_{-2i} value. Different avenues will need to be explored to understand the molecular significance of the tight-binding state of the antagonist–AT_1 receptor complexes. The distinction between this state and the initial, fast reversible state could reside at the level of the receptor conformation, its association with other proteins, or even its subcellular localization. Secondly, it appears that the initial antagonist–receptor interaction occurs in very much the same way for candesartan, EXP3174, and irbesartan and furthermore that it resembles the interaction between the surmountable antagonist losartan (S) and the receptor. As suggested by the fit between the computer-generated and the experimental data in Fig. 2, a fast reversible bimolecular process indeed appears to be sufficient to describe the losartan–receptor interaction. The dissociation and association rate constants for losartan (k_{-1s}, k_{1s}; Table 1) were set to yield a ratio of 7.5 nM. This value is very close to the K_d values of losartan obtained by competition studies with angiotensin II and [^{3}H]candesartan [3,4].

Surmountable as well as insurmountable AT_1 receptor antagonists were shown to be competitive with angiotensin II in co-incubation [5,23,25], and it was recently also shown that both types of antagonists compete with each other for binding to the receptor [25]. This is in agreement with a number of AT_1 receptor mutation studies [26,27] which indicate that both types of antagonists bind to a partially overlapping site rather deep between the membrane-spanning segments of the receptor. Based on this additional information, it may be speculated that the initial recognition of surmountable and insurmountable antagonists by the AT_1 receptor is very similar and may involve the tetrazole moiety of these ligands.

The decrease of the maximal angiotensin II-mediated response by insurmountable antagonists may be reduced or even abolished when surmountable antagonists such as losartan are present simultaneously. This phenomenon has been reported for the rabbit aortic strip contraction model [7,15,18,21,28], the pithed rat diastolic blood pressure model [28], and in isolated cell systems [19,25]. In agreement with the competitive, syntopic action of surmountable and insurmountable antagonists, the restitution of the maximal response by losartan was shown to be concentration-dependent in rabbit [11,28]. As shown in Fig. 3, such concentration-dependent restitution is also observed when measuring the angiotensin II-mediated IP production in CHO-AT_1 cells. This effect of losartan is related to its ability to produce a dose-dependent rightward shift of the insurmountable component of the inhibition curve of EXP3174 (Fig. 4). A similar shift has recently also been observed for the candesartan/losartan combination [25]. The experimental data in Figs. 3 and 4 are, here again, in good agreement with the curves that were computer-generated according to the above two-step model for insurmountable antagonists, the 1-step model for losartan, and with the ability of losartan and EXP3174 to display competitive binding to the AT_1 receptor.

These data may be of interest for the clinical situation in that they may explain the difference in efficacy for losartan (and its metabolite EXP3174) seen in the clinical setting and in previous *in vitro* and *in vivo* studies. It has previously been shown that EXP3174 is a potent, high-affinity AT_1 receptor antagonist both in *in vivo* as well as in *in vitro* experiments, with potency and efficacy values not far from those of candesartan and exceeding those of irbesartan [3,10,29]. The free (non-protein-bound) plasma concentration of losartan obtained after clinical relevant doses has, in contrast, been shown to be insufficient to antagonize the AT_1 receptor. It is therefore likely that the blood pressure reduction observed in hypertensive patients relates to the formation of EXP3174. In several clinical trials it has, though, been shown that both irbesartan and candesartan treatment results in a statistically significant better blood pressure reduction than that obtained with losartan [30–32]. As a significant interaction between losartan and EXP3174 is observed already at a losartan concentration of 30 nM even at a relatively high EXP3174 concentration, it seems plausible that the lesser clinical efficacy of losartan versus candesartan and irbesartan is caused by this interaction.

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