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COMMENTARY

Angiotensin II Type 1 Receptor Antagonists

WHY DO SOME OF THEM PRODUCE INSURMOUNTABLE INHIBITION?

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ABSTRACT. Chinese hamster ovary (CHO) cells expressing human recombinant angiotensin II type 1 (AT_1) receptors offer a useful experimental system in which antagonist binding and inhibition of AT-induced inositol mono-, bis-, and trisphosphate accumulation can be measured under identical experimental conditions. The major conclusions of the current work are: All investigated AT_1 antagonists are competitive with respect to AT. They bind to a common or overlapping binding site on the receptor in a mutually exclusive way. Reduction of the maximal angiotensin II response, i.e. insurmountable inhibition, is observed only when the cells are preincubated with candesartan, EXP3174, or irbesartan and is strictly related to the dissociation rate of the antagonist–receptor complex. On the other hand, inhibition by losartan is fully surmountable by AT, and its dissociation is very rapid. With respect to the binding kinetics, the antagonist–receptor complex can adopt a fast and a slow reversible state. The equilibrium between both states, which is dependent upon the nature of the antagonists, determines the extent of insurmountable inhibition. Consequently, the dissociation rate of the different antagonists correlates with the amount of insurmountable inhibition. In addition to the relatively slow dissociation of candesartan, reassociation to the receptor, which is measurable in CHO-AT_1 cells, likely contributes to its long-lasting blood pressure lowering effect *in vivo*. BIOCHEM PHARMACOL **60**;11:1557–1563, 2000. © 2000 Elsevier Science Inc.

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

It is well documented that the AT† type 1 receptor plays a pivotal role in cardiovascular homeostasis; therefore, there is considerable interest in developing selective antagonists for this receptor for the clinical treatment of hypertension and congestive heart failure [1, 2]. The inhibitory properties of synthetic non-peptide AT_1 receptor antagonists traditionally are assessed by measuring AT-induced contraction of rabbit aortic rings or strips. Whereas preincubation with some antagonists (denoted as surmountable antagonists), such as losartan, only produces parallel rightward shifts of the angiotensin concentration–response curve, others (denoted as insurmountable or non-competitive antagonists) also cause a depression of the maximal response of AT. The degree by which these antagonists depress the maximal response is variable, ranging from a partial decline by irbesartan, valsartan, and EXP3174 (the active metabolite of losartan) up to an almost complete inhibition by GR117289, KRH-594, EXP597, and candesartan [3–9]. Several theories have been proposed to explain this particular behavior of AT_1 receptor antagonists. These theories include the presence of allosteric binding sites on the receptor [10], slowly interconverting receptor conformations [11, 12], slow removal of the antagonist from tissue compartments, cells, or matrix surrounding the receptor [13], coexistence of different receptor subpopulations, the ability of antagonists to modulate the amount of internalized receptors [5], or two antagonist-induced receptor states with fast and slow dissociation [14].

There is a substantial need for the comparison of radioligand-binding properties of such antagonists with their *in vitro* and *in vivo* inhibitory properties to unravel the underlying molecular mechanism(s) of insurmountable antagonism. Moreover, these findings might shed light on the long-lasting blood pressure lowering effect of some of these antagonists. In the present commentary we discuss CHO cells expressing a human recombinant AT_1 receptor as a model system in which both the radioligand binding and the receptor function (i.e. AT-induced IP production) can be measured under identical experimental conditions [15]. The usefulness and/or limitations of this model will be discussed in relation to the following questions/topics:

1. Can CHO-AT_1 cells be used to study insurmountable antagonism?
2. Are insurmountable AT_1 antagonists competitive with AT? Do AT_1 antagonists bind to a common binding site of the receptor?
3. Is insurmountable antagonism related to long-lasting inhibition of AT_1 receptor function?

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† *Abbreviations*: AT, angiotensin II; CHO-AT_1 cells, Chinese hamster ovary cells expressing human AT_1 receptors; and IP, inositol mono-, bis- and trisphosphates.

TABLE 1. Concentration–response curves of AT-induced IP accumulation: Effect of AT_1 antagonist preincubation for 30 min at 37°

Antagonist	Concentration (nM)	EC_{50} (nM)	E_{max} (% of control)
Medium only	0	3.4 ± 0.7	100 ± 1.8
Candesartan	0.5	5.3 ± 0.7	57 ± 2.6*
	1.5	†	6 ± 2.1*
	5	†	7 ± 1.3*
Candesartan + 1 μM losartan	5	140 ± 21*	78 ± 1.1*
EXP3174	0.5	5.6 ± 1.2	64 ± 6.6*
	1.5	5.3 ± 1.1	46 ± 4.8*
	5	13.8 ± 1.9*	29 ± 2.4*
EXP3174 + μM losartan	5	113 ± 27*	81 ± 1.7*
Irbesartan	10	15.1 ± 4.7	71 ± 5.2*
	30	29.4 ± 10.8	73.4 ± 9.4*
	100	111 ± 36.2*	73.2 ± 9.6*
Irbesartan + 1 μM losartan	10	86 ± 38*	93 ± 3.1
Losartan	100	25.4 ± 7.6*	98 ± 5.1
	300	61.3 ± 15.0*	95 ± 3.6
	1000	181.7 ± 26.1*	92 ± 5.3

The EC_{50} and E_{max} (maximal stimulation) values are the averages ± SEM of at least three independent experiments (duplicate determinations). The E_{max} values are expressed as percent of the maximal AT response after preincubation with medium only. Reprinted with permission from *Br J Pharmacol* **126:** 1057–1065, 1999 [Copyright (1999) Nature Publishing Group] [Ref. 3] and from *Biochem Pharmacol* **59:** 927–935, 2000 [Copyright (2000) Elsevier Science] [Ref. 16].

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

†No accurate calculation of EC_{50} values is possible.

4. Why do insurmountable antagonists depress the maximal AT responses to a different degree?
5. Future questions and perspectives.

CAN CHO-AT_1 CELLS BE USED TO STUDY INSURMOUNTABLE ANTAGONISM?

CHO cells have been stably transfected with the gene coding for the human AT_1 receptor. AT_1 receptor function in adherent intact cells is detected by measuring the AT-induced increase of IP production, a transient rise of intracellular calcium concentration, and an acceleration of the extracellular acidification [3]. The AT concentration–response curves of these assays yield EC_{50} values comparable to those for the contractile response in rabbit aortic strips or rings and rat portal veins [3]. The IP accumulation in the presence of 10 mM LiCl, which increases linearly for at least 10 min, has been selected to further investigate the pharmacological properties of AT_1 antagonists [3]. As in contractile studies, the CHO-AT_1 cells are first preincubated with antagonist for 30 min (at 37°), after which IP accumulation induced by a 5-min incubation with AT is determined. Under these conditions, the investigated AT_1 antagonists cause, along with a rightward shift of the concentration–response curve, a depression of the maximal AT response (E_{max}) to a varying degree: 94% for candesartan, 71% for EXP3174, 27% for irbesartan, and not detectable for the typical surmountable antagonist losartan (Table 1 and Fig. 1). Comparable full and partial insurmountable antagonism, with the same degree of depression of the E_{max}, are reported for aorta contraction studies [4, 8, 9]. In parallel, the inhibition of [^{3}H]AT binding to cell surface receptors (i.e. extracted by mild acid treatment of adherent CHO-AT_1 cells) is determined after preincubation with AT_1 antagonists [3]. Whereas candesartan causes a substantial decline of the B_{max}, losartan and irbesartan are only capable of increasing the K_D for [^{3}H]AT without a change

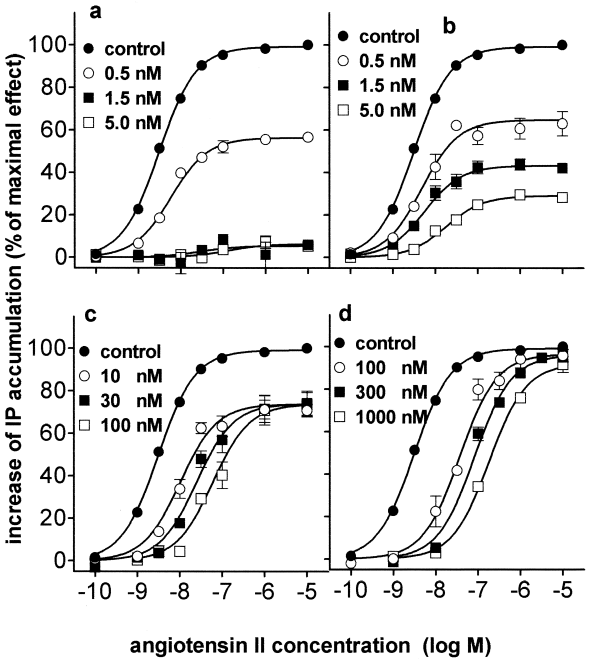

FIG. 1. Concentration–response curves of AT-mediated IP production induced by a 5-min incubation of CHO-AT1 cells after preincubation for 30 min with candesartan (a), EXP3174 (b), irbesartan (c), or losartan (d) at the indicated concentrations. Reprinted with permission from *Br J Pharmacol* 126: 1057–1065, 1999. Copyright (1999) Nature Publishing Group. [Ref. 3].

TABLE 2. [^{3}H]AT saturation binding to CHO-AT_1 cell surface receptors (extracted by mild acid treatment with ice-cold 50 mM glycine/125 mM NaCl buffer at pH 3) after preincubation with AT_1 antagonists for 30 min

Condition	Apparent K_D (nM)	B_{max} (fmol/10^6 cells)
Control	2.12 ± 0.16	19.7 ± 2.3
0.25 nM Candesartan	2.83 ± 0.57	6.2 ± 0.7*
0.5 nM EXP3174	3.10 ± 0.06*	8.0 ± 0.2*
5 nM Irbesartan	5.20 ± 0.94*	19.1 ± 1.2
30 nM Losartan	6.7 ± 1.03*	23.2 ± 5.9

The apparent K_D and B_{max} values are the averages ± SEM of three independent experiments and are calculated by non-linear regression analysis using Graphpad Prism software. Reprinted with permission from *Br J Pharmacol* **126:** 1057–1065, 1999. Copyright (1999) Nature Publishing Group. [Ref. 3].

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

of the B_{max}. An intermediate effect is seen with EXP3174 (increase of K_D and decrease of B_{max}) (see Table 2). The inhibition of cell surface [^{3}H]AT binding coincides with a decline of AT-induced IP accumulation after preincubation with candesartan for different periods of time. As for contractile studies of rat portal veins and rabbit aorta, these data indicate that there is no receptor reserve in CHO-AT_1 cells. In other words, there is a close relationship between the number of cell surface receptors available for [^{3}H]AT binding and the extent of IP accumulation after antagonist preincubation. Thus, both biochemical assays in these cells can be used for an in-depth study of the underlying molecular mechanism(s) of insurmountable AT_1 antagonists.

ARE INSURMOUNTABLE AT_1 ANTAGONISTS COMPETITIVE WITH AT? DO AT_1 ANTAGONISTS BIND TO A COMMON BINDING SITE OF THE RECEPTOR?

To find out whether the inhibitory effect of AT_1 antagonists is competitive with AT, it is crucial that both antagonist and agonist are applied simultaneously to the receptor [14]. Such an approach is fundamentally different from contraction studies, in which the tissue is preincubated with the antagonist for variable time periods, followed by a cumulative exposure to AT. Therefore, AT concentration–response experiments in CHO-AT_1 cells have been performed in the simultaneous presence of candesartan, EXP3174, irbesartan, or losartan. In contrast to the preincubation experiments, all these antagonists were shown to produce a concentration-dependent and parallel rightward shift of the curves without affecting the maximal AT response (Fig. 2). The corresponding apparent pA_2 values calculated by Arunlakshana/Schild regression analysis [17] were 9.7, 8.9, 8.8, and 8.7 respectively. Similarly, Criscione *et al.* [7] noticed that the insurmountable antagonist valsartan decreases the maximal response in contraction experiments, whereas the maximal AT-mediated release of aldosterone measured under coincubation

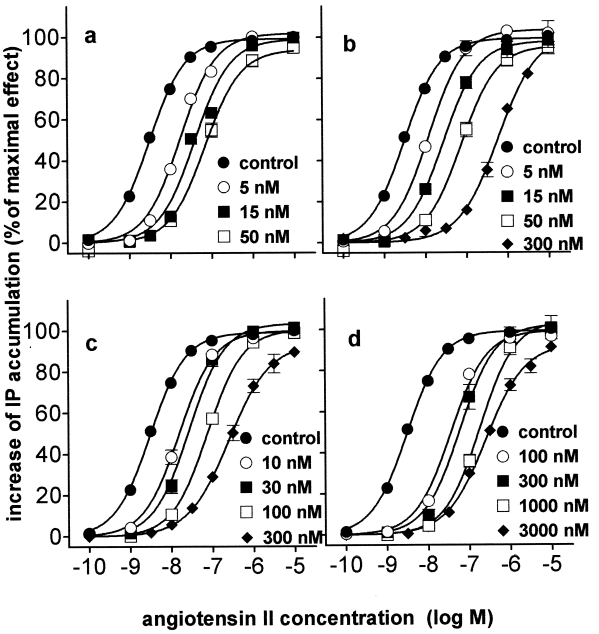

FIG. 2. Concentration–response curves of AT-mediated IP production induced by a 5-min incubation of CHO-AT_1 cells in the simultaneous presence of candesartan (a), EXP3174 (b), irbesartan (c), or losartan (d) at the indicated concentrations. Reprinted with permission from *Eur J Pharmacol* 372: 199–206, 1999. Copyright (1999) Elsevier Science. [Ref. 14].

conditions in bovine adrenal glomerulosa cells is not changed [7]. In combination with the absence of receptor reserve in the CHO-AT_1 cells (see previous section), these findings clearly establish that insurmountable AT_1 antagonists inhibit the AT response in a competitive fashion.

However, the question remains whether the different AT_1 antagonists bind to distinct sites of the receptor or to a common or overlapping binding site. Functional (IP accumulation) and [^{3}H]candesartan binding data for intact CHO-AT_1 cells allow one to address this question [16].

(a) Whereas the simultaneous presence of unlabelled candesartan, EXP3174, irbesartan, or losartan with [^{3}H]candesartan does not affect the B_{max} values from saturation binding curves, these antagonists cause a concentration–dependent increase of the apparent K_D values (Table 3).

(b) Kinetic experiments in which the dissociation rate of [^{3}H]candesartan is determined by isotopic dilution with unlabelled candesartan are not changed by the concomitant presence of EXP3174, irbesartan, or losartan [16].

(c) The insurmountable inhibition (i.e. the depression of the maximal response) after preincubation with candesartan, EXP3174, or irbesartan can be reversed by the simultaneous presence of losartan (Table 1).

Based on the above data, surmountable as well as insurmountable AT_1 antagonists are mutually competitive, i.e. they bind to a common or overlapping binding site of the AT_1 receptor.

TABLE 3. [^{3}H]Candesartan saturation binding parameters to CHO-AT$_1$ cells in the presence of unlabelled antagonists

Condition	Concentration (nM)	K_D (nM)	B_{max} (fmol/mg propein)
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 are 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. Reprinted with permission from *Biochem Pharmacol* **59:** 927–935, 2000. Copyright (2000) Elsevier Science. [Ref. 16].

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

IS INSURMOUNTABLE ANTAGONISM RELATED TO LONG-LASTING INHIBITION OF AT$_1$ RECEPTOR FUNCTION?

Recovery of AT-induced rabbit aorta strip contraction after preincubation with candesartan and EXP3174 suggested that the longevity of the antagonist–receptor complex may be responsible for the insurmountable inhibition of these antagonists. Therefore, we have investigated whether the depression of the maximal response by candesartan, EXP3174, irbesartan, and losartan in CHO-AT$_1$ cells is linked with the dissociation rate of these antagonists from the AT$_1$ receptor as assessed by three experimental approaches [15, 16]:

In the first approach, the cells are preincubated with different concentrations of AT$_1$ antagonist, then washed with fresh medium and incubated further for different time periods, after which the maximal IP response is measured by a 5-min incubation with 0.1 μM AT. As shown in Table 4, the response recovered only very slowly after candesartan pretreatment (the half-life was approximately 5 hr). In contrast, the recovery was faster for EXP3174 and still faster in the case of irbesartan and losartan. Whereas these findings are compatible with a slow dissociation of insurmountable antagonists, it does not necessarily imply that the antagonists exert their long-lasting inhibition by binding to the receptor. In this context, a slow interconversion between an inactive and active receptor conformation, much slower than the ligand binding, is proposed in a two-state model by Gero [18] and Robertson *et al.* [12] and in the related coupling model of de Chaffoy de Courcelles *et al.* [11]. To address this issue, we compared the recovery experiments with candesartan in the CHO-AT$_1$ cells (see above) with the time course of [^{3}H]candesartan dissociation as initiated by removal of unbound radioligand by washing of the cells (i.e. the same method as in functional recovery experiments) [15]. We found that the slow recovery of IP production in CHO-AT$_1$ cells completely matches the dissociation of [^{3}H]candesartan binding by washing of the cells (Tables 4 and 5). There is, therefore, no necessity to invoke additional models to explain the insurmountable behavior by candesartan other than by its relatively slow dissociation from the receptor.

TABLE 5. Dissociation rate constants for [^{3}H]candesartan (5 nM) on CHO-AT$_1$ cells

Method	k_{-1} (min^{-1})	$T_{1/2}$ (min)
Washing with medium alone	0.0015 ± 0.00010	462 ± 31
Washing + 10 μM losartan	0.0068 ± 0.0012	102 ± 18
Washing + 0.1 μM candesartan	0.0070 ± 0.0012	99 ± 17
Isotopic dilution	0.0062 ± 0.0001	112 ± 2

The k_{-1} values and corresponding half-lives ($T_{1/2}$) were obtained by linear regression analysis of the semi-logarithmic representation of the data and are the averages ± SEM of three separate experiments. Reprinted with permission from *Eur J Pharmacol* **367:** 413–422, 1999. Copyright (1999) Elsevier Science. [Ref. 15].

By performing these [^{3}H]candesartan washout experiments, we noticed that inclusion of a saturating concentration (10 μM) of losartan resulted in a 4- to 5-fold acceleration of the dissociation rate (Table 5). A similar acceleration of the recovery of the IP response was seen when losartan was included in the washout medium (Table 4). Moreover, the corresponding dissociation rate constants found using losartan-containing washout medium match

TABLE 4. Dissociation rate constants for AT$_1$ antagonists derived from the association rate of [^{3}H]candesartan binding and functional recovery experiments of CHO-AT$_1$ cells

	[^{3}H]Candesartan association		IP recovery			
			Washout + 1 μM 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	0.22 ± 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 Dulbecco's Modiefied Eagle's Medium either without or with 1 μM losartan. Reprinted with permission from *Biochem Pharmacol* **59:** 927–935, 2000. Copyright (2000) Elsevier Science. [Ref. 16].

*NM = not measurable.

with those calculated from experiments on dissociation of [^{3}H]candesartan by addition of losartan or other unlabelled antagonists. These findings can be explained by the ability of candesartan to accumulate in the washout medium and to reassociate to the receptor due to its high affinity (K_D = 51 pM [15]). Whereas it is not yet clear whether reassociation and/or accumulation of candesartan in the vicinity of the receptor occurs *in vivo* as well, these phenomena likely can contribute to its long-lasting blood pressure lowering effect. In this context, the *in vivo* effects of candesartan have been reported to persist even after its plasma concentration has become undetectable. We are currently investigating whether similar phenomena take place for other AT_1 antagonists as well.

In the last approach to determine the dissociation rate of the other (non-radiolabelled) antagonists, EXP3174, irbesartan, and losartan, we measured the slowing of the association rate of [^{3}H]candesartan after antagonist pretreatment of CHO-AT_1 cells (Table 4). Such a method has also been described by Hara *et al.* [19] and relies on a competitive interaction between these ligands. Moreover, this method is not 'biased' by eventual reassociation of the unlabelled antagonists, as this is prevented by the radioligand itself [19]. The corresponding rate constants, therefore, reflect the 'true' dissociation of the antagonist from the receptor and should be compared with the functional recovery experiment by washout of the cells in the presence of losartan. The dissociation rate constants of the investigated antagonists are independent of the different experimental procedures and are summarized in Table 5.

It is obvious that the relatively long half-life of the candesartan–and EXP3174–AT_1 receptor complex is adequate to explain the decline of the maximal induced IP production in CHO-AT_1 cells, as the incubation time with AT is only 5 min. The half-life of irbesartan is considerably shorter, but still sufficient to explain the partial decline of the maximal AT response. In agreement, the IP production induced by 10 μM AT on CHO-AT_1 cells preincubated with 100 nM irbesartan has been shown to increase when the cells are incubated for 1 to 5 min [14].

WHY DO INSURMOUNTABLE ANTAGONISTS DEPRESS THE MAXIMAL AT RESPONSES TO A DIFFERENT DEGREE?

A puzzling observation is that different AT_1 antagonists (under preincubation conditions) depress the maximal AT-mediated responses to various degrees in contractile and isolated cell studies. To improve the understanding of this phenomenon, we have quantified the insurmountable inhibition by performing antagonist concentration–inhibition experiments [14]. In these experiments, CHO-AT_1 cells are preincubated with increasing antagonist concentrations, followed by a 5-min incubation with 10 μM AT. The resulting curves are clearly biphasic, with the most potent component corresponding to insurmountable inhibition and the least potent to surmountable inhibition (Fig. 3). These results can be explained by a two-state, two-step model in which:

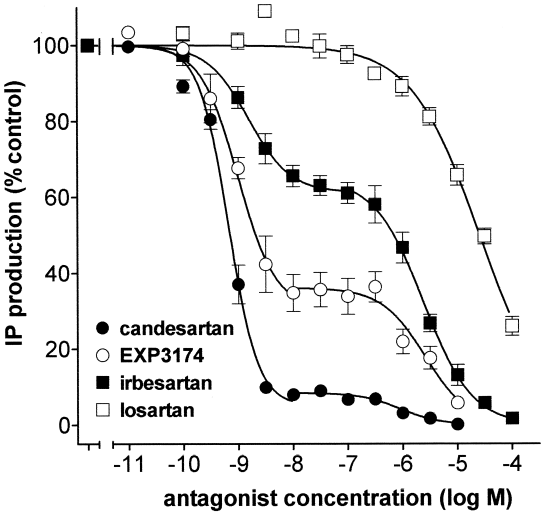

FIG. 3. Antagonist concentration–inhibition curves. The CHO-AT_1 cells were preincubated for 30 min with increasing antagonist concentrations followed by a 5-min incubation with 10 μM AT. Reprinted with permission from *Eur J Pharmacol* 372: 199–206, 1999. Copyright (1999) Elsevier Science. [Ref. 14].

- The investigated AT_1 antagonists (candesartan, EXP3174, and irbesartan) bind to the receptor in a competitive fashion.
- The antagonist–receptor complexes are able to adopt a fast reversible (ANT.R) as well as a tight-binding/slow reversible state (ANT.R*). An equilibrium between both states is established rapidly and is dependent on the nature of the antagonist, reflected by the kinetic constant k_{-2i}.
- The equilibrium between both states determines the decline by which the maximal AT response is decreased after preincubation with the antagonist.

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

We have recently generated a computer model that fitted with the experimental data (antagonist concentration–inhibition curves and AT concentration–response curves) in which $\boldsymbol{k}_{-2i}$ (i.e. the kinetic parameter corresponding to the conversion of the slow reversible into the fast reversible state) matched with experimental dissociation rates of the antagonists given in Tables 4 and 5.

FUTURE QUESTIONS AND PERSPECTIVES

As explained above, the extent of insurmountable inhibition, i.e. the decline of the maximal response after antag-

* Fierens FLP, Vanderheyden PML, De Backer JP, Thekkumkara TJ and Vauquelin G, Manuscript submitted for publication.

onist preincubation, is the result of an equilibrium between a tight/slowly reversible and a fast reversible state of the receptor. Whereas the proportion between both states is dependent upon the structure of the antagonist, their molecular significance should be explored. Among the many theoretical possibilities, the distinction between the two states might be attributed to their subcellular localization, coupling to other proteins, or different receptor conformations. In this context several authors have proposed that antagonists might control or mimic the process of AT-induced internalization of the AT_1 receptor via coated pit formation [5]. Using mild acid extraction of cell surface [^{3}H]AT binding, we were indeed able to show AT-induced receptor internalization in CHO-AT_1 cells.* In line with previous studies, AT_1 receptor internalization in these cells can be prevented by exposing the cells to a hypertonic concentration of sucrose [20]. In contrast, [^{3}H]candesartan binding to CHO-AT_1 cells is not affected by sucrose treatment. Subsequently the inhibition pattern on function and [^{3}H]candesartan binding was found to be the same on the wild-type and C-terminal truncated rat AT_{1A} receptors (previously described to have an impaired internalization capacity [21]). We have, therefore, no evidence that the tight binding of candesartan and the resulting insurmountable inhibition are related to receptor internalization via coated pit formation.

Further research should also pay attention to the precise amino acid residues on the AT_1 receptor that are involved in antagonist binding. Whereas candesartan and EXP3174 have two negatively charged groups (a carboxyl and a tetrazole moiety), their precursors, candesartan cilexetil and losartan, only have the tetrazole group. Whereas several other insurmountable antagonists have two acidic groups, their correct positioning within the binding site might be mandatory for tight binding to the receptor. Site-directed mutagenesis of certain amino acids of the AT_1 receptors will allow us to determine the residues that are involved in antagonist binding and insurmountable inhibition. Such an approach will hopefully determine whether the same basic amino acid residues serve as acceptors for the negatively charged groups on the different AT_1 antagonists.

Finally, research is necessary to find out whether reassociation as described above for candesartan, and/or "local accumulation" occurs for other AT_1 antagonists and if it contributes to the suggested long-lasting *in vivo* effect of some of them. For this purpose the direct binding properties of radiolabelled antagonists are to be compared between CHO-AT_1 cells and cells that endogenously express AT_1 receptors. Ultimately the *in vivo* receptor occupancy of AT_1 antagonists should be studied in conjunction with their blood pressure lowering effects as well as their inhibition of the pressor response to exogenous AT, and studies should determine if it contributes to the described long-lasting *in vivo* effect of some of them.

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