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Insurmountable angiotensin AT_1 receptor antagonists: the role of tight antagonist binding

Frederik L.P. Fierens *, Patrick M.L. Vanderheyden, Jean-Paul De Backer, Georges Vauquelin

Department of Molecular and Biochemical Pharmacology, Free University of Brussels (VUB), Paardenstraat 65, B-1640 Sint-Genesius Rode, Brussels, Belgium

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Abstract

Angiotensin II increased the inositol phosphates production ($EC_{50} = 3.4 \pm 0.7$ nM) in Chinese hamster ovary (CHO) cells expressing the cloned human angiotensin AT_1 receptor (CHO-AT_1 cells). Coincubation with angiotensin AT_1 receptor antagonists produced parallel rightward shifts of the concentration–response curve without affecting the maximal response. The potency order is 2-ethoxy-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benzimidazoline-7-carboxylic acid (candesartan) > 2-*n*-butyl-4-chloro-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (EXP3174) > 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]2-imidazolin-5-one (irbesartan) > of 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan). Additionally, preincubation with these antagonists depressed the maximal response, i.e., 95%, 70%, 30% of the control response for candesartan, EXP3174 and irbesartan and not detectable for losartan. Increasing the antagonist concentration or prolonging the preincubation time did not affect this depression. Furthermore, these values remained constant for candesartan and EXP3174, when the angiotensin II incubation time varied between 1 and 5 min. Our data indicate that antagonist–receptor complexes are divided into a fast reversible/surmountable population and a tight binding/insurmountable population at the very onset of the incubation with angiotensin II.

Keywords: Angiotensin II; Angiotensin AT_1 receptor; CHO (Chinese hamster ovary) cell; Inositol phosphate; Antagonist; Surmountable; Insurmountable

1. Introduction

The hypotensive effect of angiotensin AT_1 receptor antagonists is based on their ability to block angiotensin II receptors of the AT_1 subtype in vascular smooth muscle cells (Vallotton, 1987; Timmermans et al., 1992). A fair number of synthetic antagonists have been developed during the past decade and they are traditionally tested for their ability to antagonize angiotensin II-induced contraction of rabbit aortic strips. These studies include a preincubation step, in which the tissue is pre-equilibrated with the antagonist, and an incubation step which comprises the consecutive addition of increasing concentrations of angiotensin II to generate a concentration–response curve. Besides producing parallel rightward shifts of the curve, a number of these antagonists were also found to depress maximal response to angiotensin II. These findings led to the distinction between two categories of antagonists. Surmountable antagonists such as losartan only produce rightward shifts (Mochizuki et al., 1995). Insurmountable antagonists will, additionally, also depress the maximal response. Whereas this decline is almost complete for antagonists such as 2-ethoxy-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benzimidazoline-7-carboxylic acid (candesartan), it appears only to be partial for antagonists such as 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]2-imidazolin-5-one (irbesartan) and 2-*n*-butyl-4-chloro-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (EXP3174), the active metabolite of 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan) (Liu et al., 1992; Cazaubon et al., 1993; Noda et al., 1993; Mochizuki et al., 1995).

Several theories have already been advanced to explain the molecular basis for insurmountable antagonism and, especially for the frequently incomplete character of this effect. They include the presence of allosteric binding sites

* Corresponding author. Tel.: +32-2-3590265; Fax: +32-2-3590276; E-mail: ffierens@vub.ac.be

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on the receptor (Timmermans et al., 1991), slowly interconverting receptor conformations (De Chaffoy de Courcelles et al., 1986; Robertson et al., 1994), slow dissociation of the antagonist–receptor complex (Wienen et al., 1993; Dickinson et al., 1994; Olins et al., 1994; Cirillo et al., 1995; Ojima et al., 1997), slow removal of the antagonist from tissue compartments, cells or matrix surrounding the receptor (Robertson et al., 1992; Panek et al., 1995) and the ability of the antagonist to modulate the amount of internalized receptors (Liu et al., 1992).

Intact angiotensin AT_1 receptor-containing cells, either as primary cultures or as cell lines, offer the opportunity to correlate radioligand binding and functional data under the same experimental conditions. This minimizes potential sources of confusion when compared to the more traditional studies. In these studies, functional data on rabbit aortic strips are correlated with radioligand binding data on membrane preparations from often different tissues and species. Hence, studies on intact angiotensin AT_1-receptor expressing cells could lead to a better insight in the action mechanism of insurmountable antagonists. In this respect, insurmountable antagonism has effectively been shown to take place when measuring the angiotensin II-mediated inositol phosphate production in primary cultures of rabbit aortic smooth muscle cells (Dickinson et al., 1994; Panek et al., 1995) and in Chinese hamster ovary cells (CHO-K1) expressing cloned rat as well as human angiotensin AT_1 receptors (CHO-AT_1 cells) (Perlman et al., 1995; Vanderheyden et al., 1999). When the CHO-AT_1 cells were exposed to the antagonists losartan, irbesartan, EXP3174 and candesartan prior to their challenge with angiotensin II, they affected the concentration–response curve in very much the same way as in rabbit aortic strip contraction studies (Vanderheyden et al., 1999). Recent radioligand binding experiments on intact CHO-AT_1 cells further revealed that the insurmountable antagonist [^{3}H]candesartan dissociates slowly from its receptors and that this dissociation coincides with the recovery of the cell's responses to angiotensin II (Fierens et al., 1999). This agrees with earlier proposals that the insurmountable nature of at least certain of the angiotensin AT_1 receptor antagonists is related to their slow dissociation from their receptors (Wienen et al., 1993; Dickinson et al., 1994; Olins et al., 1994; Cirillo et al., 1995; Ojima et al., 1997).

In the present study, it is shown that losartan, irbesartan, EXP3174 and candesartan only produce parallel rightward shifts of the angiotensin II concentration–response curve when the agonist and antagonists are simultaneously added to the CHO-AT_1 cells, i.e., when the preincubation step with antagonist is omitted. This establishes that these antagonists inhibit the action of angiotensin II in a competitive fashion. To deal with the various degrees by which the antagonist preincubation depresses the maximal response of angiotensin II, a two-state model is proposed. In this model, the antagonist–receptor complexes are able to adopt a fast reversible as well as a tight binding state.

2. Methods

2.1. Drugs used

Candesartan (Noda et al., 1993; Shibouta et al., 1993), EXP3174 (Wong et al., 1990), losartan (Wong et al., 1990) and irbesartan (Cazaubon et al., 1993) were obtained from Astra Hässle (Mölndal, Sweden). Unlabeled angiotensin II, bovine serum and bovine serum albumin (Fraction V) were obtained from Sigma (St. Louis, MO, USA). *myo*-[^{3}H]inositol (20 Ci/mmol) was from Pharmacia/Amersham/Biotech (Buchinghamshire, UK). All other chemicals were of the highest grade commercially available.

2.2. Cell culture

Wild-type CHO-K1 were kindly donated by Dr. H. Verschueren (Pasteur Institute, Brussels, Belgium). CHO-K1 cells stably expressing the human angiotensin AT_1 receptor were obtained as described previously (Vanderheyden et al., 1999). CHO-AT_1 cells were cultivated in 75 cm^2 flasks in Dulbecco's Modified Essential Medium (DMEM) which is supplemented with 2 mM L-glutamine, 2% of a stock solution containing 5000 I.U./ml penicillin and 5000 μg/ml streptomycin (Life Technologies, Merelbeke, Belgium) and 10% fetal bovine serum (Life Technologies). The cells are grown in 5% CO_2 at 37°C until they are confluent.

2.3. Inositol phosphate accumulation

The cells were plated in 24 well plates and cultured until almost confluent. The medium was replaced by DMEM containing 10 μM unlabeled *myo*-inositol and 1 μCi/ml *myo*-[^{3}H]inositol and the cells were further grown for 20 h in 5% CO_2 at 37°C. Just before the incubation, the cells were washed 2 times with DMEM (0.5 ml per well). DMEM (400 ml) containing 10 mM LiCl was added to each well and the cells were left for 15 min at 37°C. Preincubations were initiated by addition of 50 μl DMEM/10 mM LiCl either alone (controls) or containing antagonists and proceeded at 37°C for 30 min or the indicated periods of time. Subsequent incubations were started by adding 50 μl of DMEM/10 mM LiCl either alone (basal accumulation) or containing angiotensin II and the plates were incubated at 37°C for 5 min or the indicated time periods. Coincubations started by adding 100 μl of DMEM/10 mM LiCl either alone (basal accumulation) or containing the antagonists and/or angiotensin II and the plates were incubated at 37°C for 5 min. The accumulation of inositol phosphates was determined as described by Seeuwen et al. (1988) and Vanderheyden et al. (1999). The denoted inositol phosphate accumulation represents the accumulation of the inositol mono-, bis- and triphosphates. In a previous study, the increase in angiotensin II induced inositol phosphates accumulation was linear between 0 and 10 min and then gradually levelled

off. After 30 min the cells still contained approximately 70% of the original incorporated *myo*-[^{3}H]inositol. Desensitization was only observed at longer incubation times (> 15 min) (Vanderheyden et al., 1999).

2.4. Calculations

After the preincubation with antagonist (L), it was assumed that all the receptors (R) were occupied at the onset of the subsequent incubation with angiotensin II (i.e., $[RL] = [R_{tot}]$ at $t = 0$). The time-dependency of the inositol phosphate production was simulated according to a model in which the antagonist–receptor binding was a reversible bimolecular reaction. When the maximal response was taken as unity, the response at any time is then defined as: response $= (1\text{-}e^{-k_{-1} \cdot t})$. k_{-1} is the first-order rate constant for the dissociation of $R \cdot L$ and it was assumed that angiotensin II stimulates the free receptors without delay. Responses were calculated at time intervals of 1 s and k_{-1} was obtained by iterative fitting of the equation $\sum_0^{300}(\text{response})/(300)$ to the experimental inositol phosphate production within 5 min, i.e., 0.07 times the maximal production in the presence of candesartan ($k_{-1} =$ 0.03), 0.31 for EXP3174 ($k_{-1} = 0.15$) and 0.60 for irbesartan ($k_{-1} = 0.44$). It was verified that the inositol phosphate concentration progresses almost linearly with time within the first 5 min after the addition of 10 μM angiotensin II ($r^2 = 0.992 \pm 0.001$, data not shown). The inositol phosphate production in the presence of antagonist within x s could then be calculated for each antagonist according to $\sum_0^x(\text{response})/(x)$. Calculated values are shown in Fig. 5.

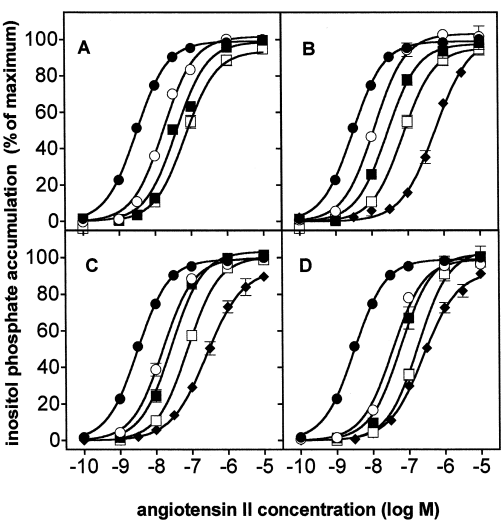

Fig. 1. Angiotensin II concentration–response curve: effect of coincubation with antagonists. CHO-AT_1 cells were incubated for 5 min at 37°C with increasing concentrations of angiotensin II (abscissa) either in the absence (●) or presence of candesartan (Panel A: ○, 3 nM; ■, 10 nM; □, 50 nM), EXP3174 (Panel B: ○, 5 nM; ■, 15 nM; □, 50 nM, ♦, 300 nM), irbesartan (Panel C: ○, 10 nM; ■, 30 nM; □, 100 nM, ♦, 300 nM) or losartan (Panel D: ○, 100 nM; ■, 300 nM; □, 1000 nM, ♦, 3000 nM). Data refer to the production of inositol phosphates above basal levels (i.e., in the absence of angiotensin II) and are expressed in percent of maximal stimulation. Data and bars are means ± S.E.M. of three experiments, three determinations each. The apparent antagonist pA_2-values, corresponding to the leftward shifts of the concentration–response curves, are listed in Section 3.

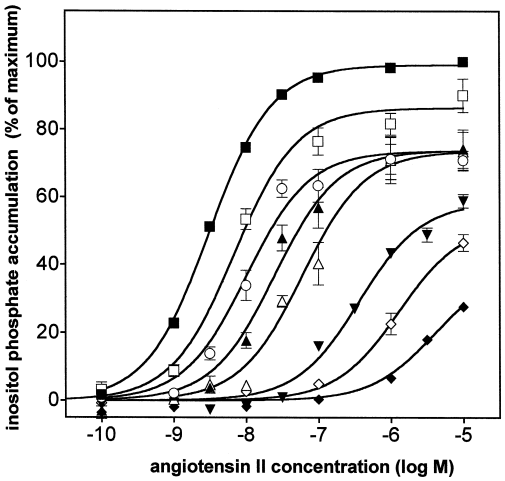

Fig. 2. Angiotensin II concentration–response curves: effect of preincubation either in the absence (■) or with different concentrations of irbesartan for 30 min at 37°C: 3 nM (□), 10 nM (○), 30 nM (▲), 100 nM (△), 300 nM (▼), 1000 nM (◇), 3000 nM (♦) after which increasing concentrations of angiotensin II (abscissa) were added and the incubation continued for 5 min. Responses are calculated as in Fig. 1.

3. Results

It was previously shown that angiotensin II produces a concentration-dependent increase of inositol phosphates in CHO-AT_1 cells (Vanderheyden et al., 1999). In these previous and in the present experiments, CHO-AT_1 cells were cultured in multiwell plates. Each well contained the same amount of cells, and angiotensin II concentration–response curves were obtained by adding different concen-

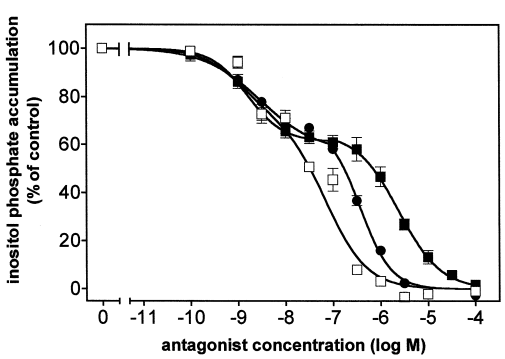

Fig. 3. Inhibition curve of irbesartan for different maximally effective angiotensin II concentrations. CHO-AT_1 cells were preincubated for 30 min at 37°C with increasing concentrations of irbesartan (abscissa) after which angiotensin II 0.1 μM (□), 1 μM (●) or 10 μM (■) was added and the incubation continued for 5 min. Responses are calculated as in Fig. 1.

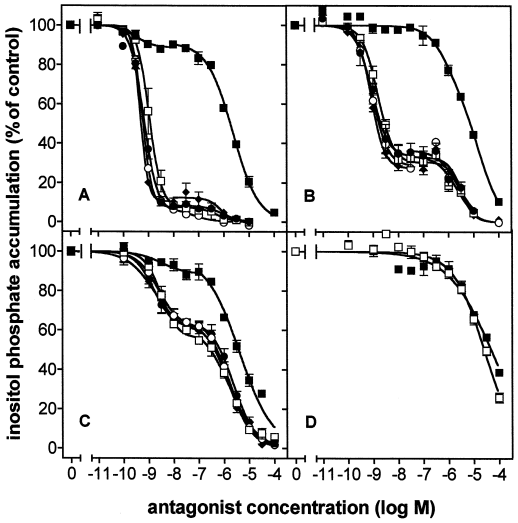

Fig. 4. Inhibition curve of candesartan (Panel A), EXP3174 (Panel B), irbesartan (Panel C) and losartan (Panel D): coincubation vs. preincubation for different periods of time. CHO-AT_1 cells were preincubated with increasing concentrations of the antagonists (abscissa) for different periods of time 10 min (□), 30 min (●), 60 min (○) and 120 min (♦) at 37°C, after which 10 μM angiotensin II was added and the incubation continued for 5 min. In the coincubation experiment (■), cells are incubated for 5 min with 10 μM angiotensin II and increasing concentrations of the antagonists (abscissa). Responses are calculated as in Fig. 1.

trations of agonist to each well. When incubated for 5 min at 37°C in the presence of 10 mM LiCl, half maximal stimulation was obtained at 3.4 ± 0.7 nM angiotensin II and maximal stimulation (at 0.1 μM angiotensin II) was 6.6 ± 0.1 times over the basal inositol phosphate level (Fig. 1). When the cells were incubated under the same conditions with a mixture of angiotensin II and the angiotensin AT_1 receptor antagonists candesartan, EXP3174, irbesartan or losartan, they all produced a rightward shift of the angiotensin II concentration–response curve without noticeably affecting the maximal stimulation (Fig. 1). This shift was concentration-dependent and the apparent pA_2-values, as calculated from the leftward shifts of the concentration–response curve according to Arunlakshana and Schild (1959), were 9.7, 8.9, 8.8 and 8.7, respectively.

When the 5 min angiotensin II incubation was preceded by a 30 min preincubation with the antagonist, the maximal response was depressed to variable degrees (Vanderheyden et al., 1999). This 'insurmountable' effect was almost complete for candesartan, intermediate for EXP3174 and irbesartan and below the level of detection for losartan. When these experiments were repeated with a wider range of irbesartan concentrations, it is shown in Fig. 2 that this antagonist decreased the maximal response by no more than 30% even though it concomitantly produced a rightward shift with increasing concentrations. These phenomena can be visualized conveniently under the form of inhibition curves. For this purpose, cells were preincubated with increasing concentrations of antagonist, followed by a challenge with a fixed concentration of angiotensin II. The resulting inhibition curves for irbesartan (Fig. 3) show that this antagonist depressed the response in a biphasic fashion. Comparison with the data shown in Fig. 2 indicated that the most potent component of the curves corresponded to insurmountable inhibition while the second, less potent component corresponded to surmountable inhibition. When

Table 1
Effect of the antagonist preincubation time on their inhibition curves (10 μM angiotensin II)

Preincubated antagonist	Preincubation time (min)	pIC_{50} surmountable	pIC_{50} insurmountable	Proportion (%) insurmountable
Candesartan	0	5.63 ± 0.04	9.49 ± 0.14	11.3 ± 1.3
	10	6.28 ± 0.13	8.96 ± 0.03	92.2 ± 2.5
	30	6.04 ± 0.11	9.17 ± 0.05	94.9 ± 3.8
	60	7.22 ± 0.71	9.24 ± 0.02	92.6 ± 1.6
	120	6.12 ± 0.36	9.32 ± 0.03	89.5 ± 1.9
EXP3174	0	5.17 ± 0.04	ND	ND
	10	5.65 ± 0.18	8.81 ± 0.02	69.9 ± 1.1
	30	5.61 ± 0.15	9.04 ± 0.05	66.6 ± 2.4
	60	5.49 ± 0.18	9.03 ± 0.04	71.4 ± 1.5
	120	5.48 ± 0.16	9.07 ± 0.03	72.5 ± 1.2
Irbesartan	0	5.38 ± 0.28	8.50 ± 0.43	11.5 ± 3.0
	10	5.78 ± 0.19	8.51 ± 0.04	44.1 ± 1.3
	30	5.62 ± 0.04	8.80 ± 0.03	38.8 ± 0.7
	60	5.78 ± 0.03	8.61 ± 0.06	39.1 ± 1.4
	120	5.80 ± 0.10	8.75 ± 0.05	41.6 ± 1.2
Losartan	0	4.31 ± 0.11	ND	ND
	30	4.59 ± 0.08	ND	ND

Apparent pIC_{50} values of the surmountable component and the insurmountable component, calculated by non-linear analysis, as well as the proportion of the insurmountable component, are given as the average ± S.E.M. of three separate determinations.
ND; not detectable.

the angiotensin II concentration was raised from 0.1 to 10 μM (Fig. 3) both components were better separated. This is due to the fact that insurmountable inhibition was independent of the agonist concentration while surmountable inhibition was competitive and hence, depended on the agonist concentration. Because of the increased separation, the concentration of angiotensin II was set at 10 μM for the ensuing experiments. Under these conditions, it is shown in Fig. 4 that the plateau between both components of the inhibition curve amounted 5–10% for candesartan, 30% for EXP3174 and 60–70% for irbesartan and 100% for losartan. These plateau values, and the apparent IC_{50}s of the surmountable and insurmountable components, were comparable for preincubation times ranging between 10 min to 2 h (Fig. 4). When the preincubation was omitted (i.e., coincubation), the inhibition curve of the 'insurmountable' antagonists (candesartan, EXP3174 and irbesartan) only displayed the surmountable component (Table 1).

Control experiments (Fig. 5) were performed to determine the effect of the angiotensin II incubation time on the extent of insurmountable inhibition. For this purpose, cells were preincubated for 30 min with 10 nM candesartan, 100 nM EXP3174 or 100 nM irbesartan (conditions corresponding to the plateau of the inhibition curves in Fig. 4) and subsequently, incubated between 1 and 5 min with 10 μM angiotensin II. Whereas the inhibition by candesartan and EXP3174 remained almost constant with the incubation time, there was a clear time-wise decrease of the inhibition by irbesartan.

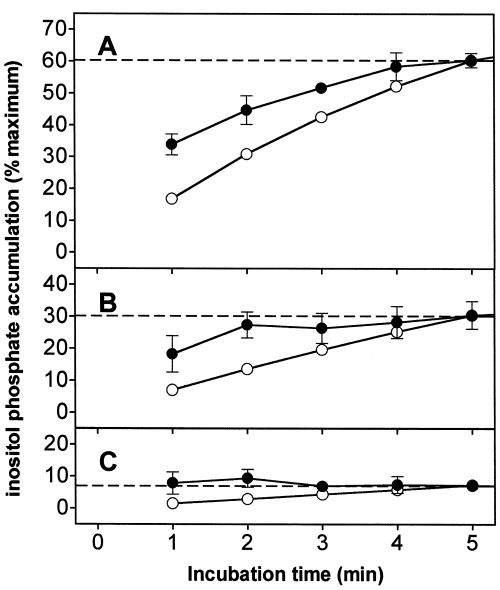

Fig. 5. The insurmountable component of the inhibition by candesartan, EXP3174 and irbesartan: effect of the incubation time with angiotensin II. CHO-AT_1 cells were preincubated for 30 min at 37°C with 100 nM irbesartan (Panel A), 100 nM EXP3174 (Panel B) or 10 nM candesartan (Panel C), after which 10 μM angiotensin II was added and the incubation continued for different periods of time (abscissa) up to 5 min. Responses (●) are calculated as in Fig. 1. The simulated inositol phosphate production after 1 to 5 min incubation (○) was obtained according to a model in which the antagonist–receptor binding is a reversible bimolecular reaction (Section 2.4).

4. Discussion

Rabbit aortic strip contraction studies have led to the distinction between surmountable and insurmountable angiotensin AT_1 receptor antagonists. Surmountable antagonists only produce rightward shifts of the angiotensin II concentration–response curve. Since, in these studies, the tissue is pre-equilibrated with the antagonist before its challenge with angiotensin II, it ensues that surmountable antagonists compete with angiotensin II for binding to the receptor and that their binding is fast reversible. Insurmountable antagonists depress the maximal response of the angiotensin II. This could indicate that they inhibit the angiotensin II-mediated response in a non-competitive fashion, e.g., by acting at an allosteric site of the receptor or by directly interfering with a cellular process that is necessary for the generation of the contractile response. Alternatively, it is also possible that they inhibit the angiotensin II-mediated response in a competitive fashion but that their antagonistic action is so slowly reversible that it cannot be reversed/overcome during the short exposure of the tissue to angiotensin II (Kenakin, 1987).

To find out whether the inhibitory effect of antagonists is competitive or not, it is mandatory for the receptors to be simultaneously exposed to the desired concentration of agonist and antagonist. This implies that two particularities that are typical for rabbit aortic strip contraction studies should be avoided: (1) the tissue is preincubated with the antagonist and (2) increasing concentrations of angiotensin II to the same tissue preparation (i.e., cumulative dosing). Thus, instead of cumulative dosing, angiotensin II should be given as single additions, one per preparation. Whereas this experimental approach is only adopted in very rare occasions for rabbit aortic strip contraction studies (Liu et al., 1992), it constitutes a standard procedure when measuring the inositol phosphate production in angiotensin AT_1 receptor-expressing cell lines (since each experiment only permits the measurement of a single response) as well as for radioligand binding studies.

To provide a better insight in these mechanisms, we compared the effect of the angiotensin AT_1 receptor antagonists candesartan, EXP3174, irbesartan and losartan on the maximal angiotensin II stimulated inositol phosphate production in CHO-AT_1 cells under pre- and coincubation conditions. The insurmountable non-peptide antagonists candesartan, EXP3174 and irbesartan were previously shown to depress the maximal angiotensin II stimulated contractile response of rabbit aortic strips to distinct degrees (Cazaubon et al., 1993; Noda et al., 1993; Mochizuki et al., 1995). When a preincubation step was included, they also depressed the maximal response to angiotensin II in CHO-AT_1 cells (Vanderheyden et al., 1999). However, it

is shown in Fig. 1 that the same antagonists produce parallel rightward shifts of the concentration response curve without affecting the maximal response under coincubation conditions. In this context, it is important to note that no receptor reserve is observed in this system, i.e., there is a close match between the K_D of cell surface [^{3}H]angiotensin II binding and the EC_{50} of angiotensin II induced inositol phosphates accumulation (Vanderheyden et al., 1999). Together with the finding that there is direct correlation between antagonist binding and functional response-inhibition (Fierens et al., 1999), this clearly establishes that the investigated antagonists in Fig. 1, inhibited the angiotensin II-mediated response in a competitive fashion. Radioligand binding studies involving pre- and coincubation experiments have already led to similar conclusions for antagonists such as candesartan and Sar^1-Ile^8-angiotensin II (Pendleton et al., 1989; Hara et al., 1995) but the present findings provide the first comparative evidence based on functional experiments on intact cell systems.

Because of their competitive nature, the insurmountable behavior of candesartan, EXP3174 and irbesartan should be related to long-lasting action. This conclusion is supported by washout experiments in which the responses of the antagonist-treated CHO-AT_1 cells to angiotensin II is only restored slowly (Vanderheyden et al., 1999). Interestingly, binding experiments with [^{3}H]candesartan on intact CHO-AT_1 cells have established that the restoration on the cell's responsiveness to angiotensin II coincides with the release of bound antagonist from the angiotensin AT_1 receptor (Fierens et al., 1999). The long-lasting action of insurmountable antagonists is therefore likely to be related to the longevity of the antagonist–receptor complex. On the other hand, the non-permanent action (or binding) of insurmountable antagonists in this and other studies (Panek et al., 1995; Hara et al., 1996; Ojima et al., 1997) argues against the occurrence of irreversible, covalent binding mechanisms. Also relevant is the work of Rang (1966) and Paton and Rang (1966) on octyltrimethylammonium induced contraction of the guinea pig. In their model, they concluded that the slow dissociation of the antagonist hyoscine, makes that the agonist can only equilibrate with the antagonist-free portion of the receptor population. Since in this system with no receptor reserve, 100% agonist receptor activation is required to produce a maximal response, this state of 'hemi-equilibrium' produces a depression of the maximal response (Kenakin, 1987).

A puzzling observation in many studies is that certain insurmountable angiotensin AT_1 receptor antagonists only depress the maximal response to a limited degree. This incomplete effect is illustrated in Fig. 2 for irbesartan. This antagonist produced an (insurmountable) decline of the angiotensin II-mediated inositol phosphate production in CHO-AT_1 cells by no more than 30% even though it concomitantly produced a rightward shift of the remaining concentration–response curve with increasing concentrations. Similar results for irbesartan were previously obtained when measuring the contraction of rabbit aortic strips (Cazaubon et al., 1993). An alternative way to illustrate and quantify these phenomena is by keeping the angiotensin II concentration fixed and by varying the antagonist concentration. In the example of irbesartan, this resulted in a biphasic inhibition curve (Fig. 3) with the most potent component corresponding to insurmountable inhibition and the least potent component corresponding to surmountable inhibition. When comparing the different angiotensin AT_1 receptor antagonists in this manner (Fig. 4), it appeared that the response was inhibited in an insurmountable fashion by up to 95% for candesartan, 70% for EXP3174, 30% for irbesartan and by less than the level of detection for losartan. The preincubation time (at least beyond 10 min) of candesartan, EXP3174 and irbesartan has little effect on the plateau values or on the apparent IC_{50}s of the surmountable and insurmountable components (Fig. 4, Table 1). When the preincubation was omitted, no insurmountable component was observed.

A reversible bimolecular reaction constitutes the simplest model to describe antagonist–angiotensin AT_1 receptor interactions. This model is only compatible with the partial insurmountable effects of irbesartan and EXP3174 in our experiments, if they undergo significant dissociation from the receptor during the 5-min incubation with angiotensin II. Computer-simulations based on an exponential, first-order dissociation of the antagonist–receptor complexes (Fig. 5) revealed that their half-lives should be 4.4 and 1.5 min to obtain, respectively 31 and 60% insurmountable inhibition of the response for a 5-min incubation with angiotensin II. This percentage of insurmountable inhibition should gradually increase when the incubation time is shortened. However, control experiments (Fig. 5) indicate that the inhibition was well below the predicted values for both antagonists at the shortest incubation times. This was not compatible with the hypothesis of a simple reversible bimolecular reaction. On the other hand, the almost steady inhibition by EXP3174 in these experiments was particularly interesting since it suggested that, at the very onset of the incubation with angiotensin II, antagonist–receptor complexes were already divided into a fast reversible/surmountable population (i.e., $L \cdot R$) and a tight binding/insurmountable population (i.e., $L \cdot R^*$).

The experiments in which the antagonist concentration and the preincubation time were allowed to vary (Fig. 4) clearly indicated that angiotensin AT_1 receptor antagonists only depressed the maximal response to a limited degree. This suggested that an equilibrium between $L \cdot R$ and $L \cdot R^*$ was reached after some time. The extent of insurmountable antagonism could provide information about this equilibrium when the proportion of $L \cdot R^*$ remained sufficiently constant during the incubation with angiotensin II. This applied to EXP3174 and candesartan (Fig. 5B and C), whose respective $L \cdot R^*/L \cdot R$ ratios at

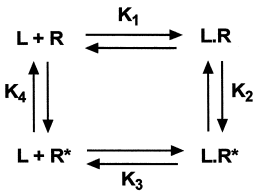

Fig. 6. General proposition of a two-state model for tight antagonist binding, with L = antagonist, R = native receptor and R* = receptor in a state of tight binding. The equilibrium between R and R* is dictated by their relative affinities for the antagonist (i.e., equilibria K_3, K_4, K_1).

equilibrium should be close to 2.3 (for 70% insurmountable inhibition) and 10–20 (for 90–95% insurmountable inhibition). Such evaluation becomes more difficult when, such as for irbesartan, there is a substantial disappearance of L · R* with time.

The relationship between L · R and L · R* can be described by a general model similar to the one proposed for a two-state mechanism of receptor activation (Leff, 1995) (see Fig. 6). This opens the possibility of a two-state mechanism (Severne et al., 1986) in which the initial binding of the ligand (L) to R is fast and reversible and the antagonist subsequently induces the receptor to adopt a tight binding state (i.e., equilibria K_1, K_2) as well as a two-state model (Kenakin, 1987) in which both receptor states are in equilibrium and, in the presence of an antagonist, their proportion is dictated by their relative affinities for that antagonist (i.e., equilibria K_3, K_4, K_1).

At present, it is unclear whether the difference between L · R and L · R* resides at the level of the receptor conformation, its association with other proteins or even its sub-cellular localization. The ability of antagonist–angiotensin AT_1 receptor complexes to adopt different conformations has already been evoked by several authors (Gero, 1983; Robertson et al., 1994; Renzetti et al., 1995; Balmforth et al., 1997). However, as none of the antagonists affects the basal inositol phosphate levels in CHO-AT_1 cells, it is unlikely that L · R or L · R* represent an 'active' conformation. Alternatively, it is well known that seven trans-membrane receptors may adopt a 'tight agonist binding' conformation when coupled to G proteins (De Lean et al., 1980; Severne et al., 1986; Convents et al., 1987). It can therefore not be excluded that L · R and L · R* are differently associated to certain other proteins or, as recently described for δ-opioid and angiotensin II receptors (Monnot et al., 1995; Cvejic and Devi, 1997), that they even exist as mono- and dimers. In these cases, an additional variable would have to be introduced and a ternary complex model would apply. Finally, the possibility arises that L · R represents a membrane-associated state of the receptor and that L · R* has undergone internalization in the cell. This possibility is based on the proposal by Liu et al. (1992) that the insurmountable effect of non-peptide angiotensin AT_1 receptor antagonists may be related to their ability to control the amount of internalized AT_1 receptors. In this vein, it has also been shown in a number of reports that the binding of radiolabeled angiotensin AT_1 receptor antagonists to intact cell systems is resistant to a mild acid treatment (Crozat et al., 1986; Conchon et al., 1994). Based on the similarity with the binding of angiotensin II, it was concluded that internalized antagonist–angiotensin AT_1 receptor complexes may account for this resistant binding.

In conclusion, the present findings establish that insurmountable angiotensin AT_1 receptor antagonists such as candesartan, EXP3174 and irbesartan inhibit the action of angiotensin II in a competitive fashion. To act insurmountably, they need to bind to the receptor before the exposure of the cells or tissue angiotensin II. The present data also suggest that the antagonist–receptor complexes are able to adopt a fast reversible and a tight binding state and that the equilibrium between both states is dependent on the nature of the antagonist. This equilibrium, along with the dissociation rate of the tight binding complex will determine the extent by which the antagonist diminishes the maximal angiotensin II-mediated response. Further research is needed to clarify the molecular characteristics and the sub-cellular distribution of the fast reversible and tight antagonist binding states of the angiotensin AT_1 receptor.

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