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Binding of the antagonist [^{3}H]candesartan to angiotensin II AT_1 receptor-tranfected Chinese hamster ovary cells

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Abstract

Binding of the non-peptide angiotensin II AT_1 antagonist [^{3}H](2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid ([^{3}H]candesartan) to human angiotensin II AT_1 receptor-transfected Chinese hamster ovary (CHO-AT_1) cells was inhibited to the same extent by angiotensin II and non-peptide angiotensin II AT_1 antagonists. No binding was observed in control CHO-K_1 cells. Dissociation was slow ($k_{-1} = 0.0010 \pm 0.0001$ min^{-1}) after removal of the free [^{3}H]candesartan but increased 5-fold upon addition of supramaximal concentrations of angiotensin II AT_1 antagonists. Angiotensin II responses recovered equally slow from candesartan-pretreatment. When washed and further incubated, these angiotensin II responses also recovered more rapidly in the presence of 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan), indicating that unlabelled ligands prevented reassociation. [^{3}H]candesartan saturation binding experiments required a long time to reach equilibrium. Therefore, the equilibrium dissociation constant ($K_d = 51 \pm 8$ pM) was calculated from the association and dissociation rate constants. Our findings indicate that the insurmountable nature of candesartan in functional studies is related to its slow dissociation from the receptor.

Keywords: Angiotensin II; [^{3}H]Candesartan; Non-peptide antagonist; Dissociation; Recovery; Reassociation

1. Introduction

Angiotensin II, the effector peptide of the renin–angiotensin system, produces a variety of biological actions including vascular smooth muscle contraction and growth of smooth muscle cells and cardiac myocytes (Timmermans et al., 1992). Angiotensin II receptor subtypes are members of the seven transmembrane guanyl nucleotide-binding protein (G-protein)-coupled receptor superfamily (Bumpus et al., 1991) and were divided into the AT_1 and AT_2 subtypes (Iwai and Inagami, 1992; Kakar et al., 1992).

As the angiotensin II AT_1 receptor plays a major role in the regulation of cardiovascular homeostasis, there has been much interest in developing angiotensin II type I antagonists for the treatment of hypertension and congestive heart failure (Vallotton, 1987). Their ability to antagonize the angiotensin II-mediated contraction of isolated rabbit aortic rings/strips, a system with very small receptor reserve (Zhang et al., 1993), is traditionally invoked to describe their properties. These studies revealed the existence of both surmountable and insurmountable angiotensin II type I antagonists. Whereas both produce parallel rightward shifts of the angiotensin II concentration–response curves, the maximal response is unaffected by surmountable antagonists such as losartan (Mochizuki et al., 1995) but depressed by insurmountable antagonists. The extent of this decline varies from one antagonist to another (Noda et al., 1995; Mochizuki et al., 1995). The molecular basis for insurmountable antagonism is still poorly understood and, although many authors favor a link between this phenomenon and slow dissociation, it has also been speculated that it could be related to the occurrence of allosteric binding sites on the receptor (Timmermans et al., 1991), the existence of slowly interconverting receptor conformations (De Chaffoy de Courcelles et al., 1986; Robertson et al., 1994), 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 affect the amount of internalized receptors (Liu et al., 1992).

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To unravel the molecular mechanisms that are responsible for insurmountable antagonism, a limited number of reports have compared the binding of (radiolabelled) antagonists with their functional properties. However, the differences between the experimental conditions for both types of studies are often outspoken and include the use of membranes for radioligand binding vs. intact tissues for functional studies as well as the use of different tissues and different species. Intact human angiotensin II AT_1 receptor-containing cells, either as primary cultures or as cell lines, offer the opportunity to perform both types of studies on the same system and, hence, to minimize potential sources of confusion. In this respect, a variety of angiotensin II AT_1 receptor-mediated responses can be measured on isolated cells and the insurmountable behavior of angiotensin II type I antagonists has already been noticed by measuring their effects on the angiotensin II-mediated increase in phosphoinositide hydrolysis, transient rise in the cytosolic Ca^{2+} level and increase in the extracellular acidification rate (Chang et al., 1992; Dickinson et al., 1994; Panek et al., 1995; Perlman et al., 1995; Balmforth et al., 1997).

We have recently investigated the effects of angiotensin II type I antagonists in Chinese hamster ovary (CHO) cells expressing the cloned human angiotensin II AT_1 receptor (CHO-AT_1 cells) by measuring the accumulation of inositol phosphates (Vanderheyden et al., 1998). When the cells were exposed to the antagonists prior to their challenge with angiotensin II, these antagonists produced parallel rightward shifts of the agonist concentration–response curve (for losartan) or depressed the maximal response to various degrees (candesartan, EXP3174, irbesartan). Their potencies (candesartan > EXP3174 > irbesartan > losartan) were comparable to those observed in rabbit aortic ring contraction studies. Candesartan, which produced an almost complete depression of the response, also produced a long-lasting inhibition in both kinds of studies (Vanderheyden et al., 1998). In the present study, we show that the binding of [^{3}H]candesartan to its receptors is highly specific in CHO-AT_1 cells. Its dissociation is very slow and coincides with the recovery of the cell's response to angiotensin II. Interestingly, the dissociation and the recovery are both enhanced about 4 to 5-fold in the presence of losartan. These findings permit a more precise determination of the molecular mechanisms that are behind the insurmountable nature of antagonists such as candesartan.

2. Materials and methods

2.1. Materials

2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid (Candesartan; Shibouta et al., 1993; Noda et al., 1995), 2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl) biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (EXP3174; Wong et al., 1990), 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan; Wong et al., 1990) and 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]2-imidazolin-5-one (irbesartan; Cazaubon et al., 1993) were obtained from Astra Hässle (Mölndal, Sweden). Angiotensin II and Bovine serum albumin (BSA, Fraction V) were obtained from Sigma (St. Louis, MO, USA). [^{3}H]Angiotensin II (50 Ci/mmol) was obtained from New England Nuclear (Boston, USA) and *myo*-[^{3}H]inositol (20 Ci/mmol) was from Pharmacia/ Amersham/ Biotech (Buchinghamshire, UK). [^{3}H]candesartan (22 Ci/mmol) was kindly provided by Takeda Chemical Industries (Osaka, Japan). All other chemicals were of the highest grade commercially available.

2.2. Cells

Wild-type Chinese hamster ovary cells (CHO-K1) were kindly donated by Dr. H. Verschueren (Pasteur Institute, Brussels, Belgium). CHO-K1 cells stably expressing the human angiotensin II AT_1 receptor (denoted as CHO-AT_1 cells) were obtained as described by Vanderheyden et al. (1998). CHO-K1 and CHO-AT_1 cells were cultured in 75 cm^2 flasks in Dulbecco's modified essential medium (DMEM) which was supplemented with L-glutamine (2 mM), 2% of a stock solution containing 5000 I.U./ml penicillin and 5000 μg/ml streptomycin (Life Technologies, Merelbeke, Belgium), 1% of a solution of MEM containing non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum (Life Technologies, Merelbeke, Belgium). The cells were grown in 5% CO_2 at 37°C until they were confluent.

2.3. Radioligand binding

Cells were plated in 24-well plates and cultured until confluence. Before the experiment, the cells were washed three times with 0.5 ml per well of DMEM at room temperature. After removal of the medium, 400 μl binding DMEM was added and the plate was then left for 15 min at 37°C. For saturation binding assays cells were incubated with increasing concentrations [^{3}H]candesartan (final concentrations between 0.15 and 15 nM) in a final volume of 0.5 ml at 37°C for 5 to 180 min.

For competition binding assays 50 μl of buffer or 50 μl of buffer containing increasing concentrations of unlabelled antagonist was added. After 30 min, 50 μl of buffer containing [^{3}H]candesartan (final concentration 1.1 nM) or [^{3}H]angiotensin II (final concentration 1.0 nM) was added, and the cells were further incubated for 30 min at 37°C.

For dissociation experiments, cells were incubated with [^{3}H]candesartan for 30 min In the first procedure, dissocia-

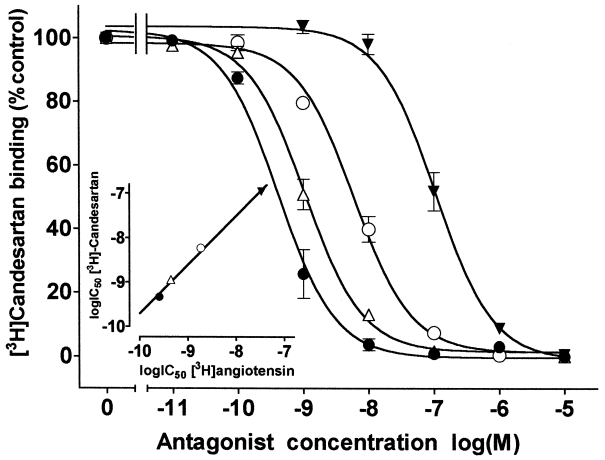

Fig. 1. [^{3}H]Candesartan binding to CHO-AT_1 cells: competition with unlabelled angiotensin II AT_1 receptor ligands. Cells were pre-incubated 30 min in the absence (control binding) or presence of increasing concentrations (abscissa) of candesartan (●), EXP3174 (△), irbesartan (○) and losartan (▼). Subsequently cells were incubated for 30 min at 37°C with 1.1 nM [^{3}H]candesartan. The corresponding IC_{50} values are given in Table 1. Inset: comparison between the inhibitory potencies ($logIC_{50}$ values) on [^{3}H]Candesartan binding and cell surface [^{3}H]Angiotensin II binding (acid wash fraction).

tion was initiated by the addition of 0.1 μM candesartan, 0.1 μM angiotensin II or 10 μM losartan to the buffer (final concentrations) and the incubation was extended for various periods of time up to 4 h. In the second procedure, dissociation was initiated by washing twice with DMEM at 37°C followed by further incubation in buffer only or in buffer containing different concentrations of candesartan, EXP3174, irbesartan or losartan.

At the end of the incubation, the 24-well plates were placed on ice and the cells were washed three times with phosphate-buffered saline buffer (containing 0.132 g/l $CaCl_2 \cdot H_2O$, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 8 g/l NaCl and 1.44 g/l $Na_2HPO_4 \cdot 2H_2O$) at 4°C, pH 7.4.

The cell surface [^{3}H]angiotensin II binding was eluted by mild acid treatment: i.e., a 5-min incubation with 0.5 ml ice-cold 50 mM glycine buffer (pH 3) containing 125 mM NaCl. This step was repeated and the radioactivity in the pooled fractions was counted after addition of 7 ml of scintillation liquid (Optifase of Wallac, Turku, Finland) for 3 min in a liquid scintillation counter. To measure internalized [^{3}H]angiotensin II, 0.5 ml 1 M NaOH was added to the acid-treated cells and the solubilized radioactivity counted in 3 ml scintillation liquid. [^{3}H]candesartan binding was measured after addition of 0.5 ml 1 M NaOH. Non-specific binding, as determined in the presence of 1 μM unlabeled angiotensin for [^{3}H]angiotensin II binding or 0.1 μM candesartan for [^{3}H]candesartan binding, was subtracted from the total binding to yield specific binding. The calculation of the binding parameters from the association and dissociation binding curves (k_{obs} and k_{-1}-values), competition binding curves (IC_{50} values) and saturation binding curves (apparent K_d and B_{max} values) was performed using GraphPad Prism. Data and bars were means ± S.E.M. of three experiments, three determinations each, refer to specific binding and data were presented as percentage of control (i.e., 30 min incubation with 1.1 nM [^{3}H]candesartan).

2.4. 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 unlabelled 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 and finally washed two times with DMEM (1 ml per well).

For the study of the antagonistic time-dependency of candesartan, the cells were pre-incubated with 450 μl DMEM containing 10 mM LiCl and 1.5 nM candesartan for different periods of time and subsequently 50 μl of DMEM containing 10 mM LiCl was added, either alone (basal accumulation) or containing 0.1 μM angiotensin II. The plates were further incubated at 37°C for 5 min.

To start the recovery experiments, cells were preincubated for 30 min at 37°C with 0.5 ml DMEM alone (as control) or DMEM containing 1.5 nM candesartan. Then the cells were washed three times with DMEM and further incubated at 37°C for 0 to 4 h with 0.5 ml DMEM alone or DMEM containing 100 μM losartan. Subsequently the cells were washed three times with 0.5 ml DMEM/well, left for the indicated periods of time at 37°C, washed three times again and left for 15 min at 37°C in 0.4 ml/well DMEM containing 10 mM LiCl. Incubations started by adding 50 μl of DMEM containing 10 mM LiCl either alone (basal accumulation) or containing 0.1 μM 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). Briefly, the plates were placed on ice at the end of the incubation, the

Table 1
Potency of inhibition of [^{3}H]candesartan and [^{3}H]angiotensin II binding

Compound	Candesartan	Exp3174	Irbesartan	Losartan
	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)
[^{3}H]candesartan	0.50 ± 0.10	1.2 ± 0.2	6.0 ± 1.0	115 ± 24
[^{3}H]angiotensin II (Acid)[a]	0.26 ± 0.05	0.4 ± 0.1	1.9 ± 1.1	34 ± 7
[^{3}H]angiotensin II (NaOH)[a]	0.31 ± 0.09	0.6 ± 0.2	1.6 ± 0.8	24 ± 12

[a] For the [^{3}H]angiotensin II binding both mild acid wash fraction (Acid) and the remaining binding (NaOH) were collected and measured. The IC_{50} values are calculated by nonlinear regression analysis and are means ± S.E.M. of 3–4 independent experiments (three determinations each).

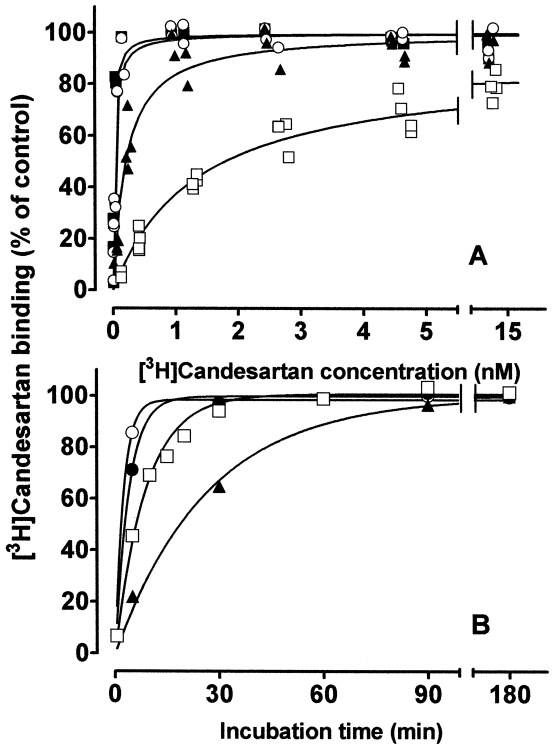

Fig. 2. Specific binding of [^{3}H]candesartan to CHO-AT$_1$ cells: effect of radioligand concentration and incubation time. (A) Saturation binding: i.e., representation of the data as a function of the [^{3}H]candesartan concentration. Corresponding apparent K_d values are 1.00 ± 0.07 nM for 5 min (□), 0.2 ± 0.02 nM for 30 min (▲), 0.015 ± 0.01 nM for 90 min (■) and 0.02 ± 0.01 nM for 180 min (○). (B) Time course of association: i.e., representation of the same data as a function of the incubation time: 0.5 nM (▲), 1.5 nM (□), 3.0 nM (●) and 15.0 nM (○).

medium was aspirated and 0.75 ml of 10 mM formic acid at 4°C was added to each well. After 30 min this solution was mixed with 3 ml of 5 mM NH_4OH and applied to a column containing 1 ml Bio-Rad anion exchange resin (AG 1×8, formate form, 200–400 mesh). Free inositol was eluted by 3 ml of 0.1 M formic acid and inositol mono-, bis- and trisphosphates were eluted with 3 ml of 0.1 M formic acid containing 0.8 M ammonium formate. The latter eluate was collected into scintillation vials, 10 ml of scintillation liquid (Ultima-flo-AF, Packard, Groningen, The Netherlands) was added and the vials were counted for at least 3 min in a liquid scintillation counter. Data refer to production of inositol phosphates above basal levels (i.e., in the absence of angiotensin II) and were expressed in percentage of control (same treatment but with omission of candesartan in the preincubation step). All data and bars were means ± S.E.M. of three experiments, three determinations each.

2.5. Computer simulations

Computer simulations (Fig. 7) were performed by repeatedly integrating the following differential equations over periods of time (t) of 0.01 min; no re-association: $d[RL]/dt = -k_{-1}[RL]$; re-association: $d[RL]/dt = -d[R]/dt = -d[L]/dt = -k_{-1}.[R.L] + k_1[R][L]$; re-association with accumulation of the radioligand: $d[RL]/dt = -d[R]/dt = -d([L]/n)/dt = -k_{-1}[RL] + k_1[R][L]$ [R]. [L] and [RL] were the concentration of free receptor, local radioligand and receptor–radioligand complex. [L] exceeded the total concentration of released radioligand by a factor n. Starting conditions were: [RL] = 0.12 nM, $[L] = [R] = 0$ M. The association rate constant, k_1 of $9.80 \pm 0.4 \times 10^{-7}$ M − 1 min^{-1} was calculated from the equation $k_1 = k_{obs} - k_{-1}/[L]$ from the kinetic experiments shown in Figs. 3 and 5.

3. Results

It was previously shown that incubation of plated CHO-AT$_1$ cells for 30 min at 37°C with 1.5 nM candesartan produces an almost complete decline in the angiotensin II-mediated inositol phosphate accumulation (Vanderheyden et al., 1998). When competition binding experiments with [^{3}H]candesartan were performed under the same con-

Table 2
[^{3}H]candesartan dissociation rate constants (k_{-1}) ($\times 10^{-3}$ min^{-1}), determined by isotopic dilution with or without subsequent washing

Pre-incubation conc. [^{3}H]candesartan (nM)	Without washing		With washing		
	0.5	5.0	0.5	1.5	5.0
Buffer	n.a.	n.a.	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Candesartan 10^{-7} M	6.1 ± 0.8	6.2 ± 0.1	7.0 ± 0.5	5.0 ± 0.3	7.0 ± 1.2
Losartan 10^{-5} M	5.3 ± 0.6	5.3 ± 0.5	5.5 ± 1.4	4.6 ± 0.1	6.8 ± 1.2
Angiotensin II 10^{-7} M	4.2 ± 0.8	4.1 ± 0.8	3.8 ± 0.7	3.7 ± 0.1	4.5 ± 0.3
EXP3174 10^{-7} M				4.9 ± 0.5	
Irbesartan 10^{-6} M				4.7 ± 0.1	
0.1% Bovine serum albumin				2.5 ± 0.5	

n.a.: not applicable.
The k_{-1} values are obtained by linear regression analysis of the semi-logarihmic representation of the data and are means ± S.E.M. of three experiments (three determinations each).

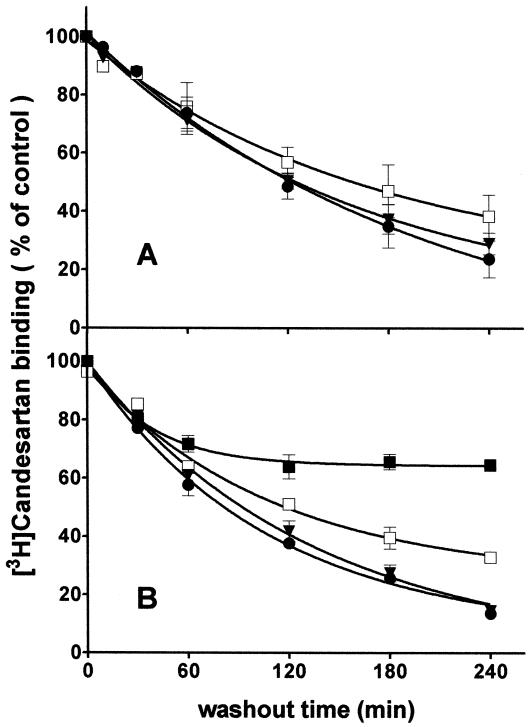

Fig. 3. Time course of [^{3}H]candesartan dissociation: effect of isotopic dilution. CHO-AT_1 cells were preincubated for 30 min with 5.0 nM [^{3}H]candesartan. For panel A: dissociation of the radioligand was initiated by addition of 0.1 μM candesartan (●), 100 μM losartan (▼) or 0.1 μM angiotensin II (□) to the medium. For panel B: dissociation of the radioligand was initiated by washing of the cells and replacement with fresh medium either alone (■) or medium containing 0.1 μM candesartan (●), 100 μM losartan (▼) or 0.1 μM angiotensin II (□). Data refer to specific binding and are expressed in percentage of control binding (preincubation with radioligand (panel A) or with subsequent washing (panel B) only). The corresponding k_{-1} values, obtained by linear regression analysis of the semi-logarithmic representation of the data are given in Table 2.

ditions, all the tested angiotensin II AT_1 receptor ligands produced a dose-dependent decline in the binding up to 95–98% of the total binding (Fig. 1). The potency order of the antagonists (candesartan > EXP3174 > irbesartan > losartan) is similar to the one observed for competition binding experiments with [^{3}H]angiotensin II (Table 1). The IC_{50} values of the [^{3}H]angiotensin and [^{3}H]candesartan competition binding curves are very similar and show a high degree of correlation ($r^2 = 0.99$) (Fig. 1 inset). In contrast, no displaceable binding of [^{3}H]candesartan could be detected on wild type CHO-K1 cells (data not shown). These data indicate that [^{3}H]candesartan binds with high specificity to the angiotensin II AT_1 receptors in CHO-AT_1 cells.

[^{3}H]Candesartan saturation binding experiments were performed for four different incubation times: 5, 30, 90 and 180 min (Fig. 2). Whereas the maximal binding is similar for the four incubation times ($B_{max} = 79 \pm 5$ fmol/10^6 cells ($n = 17$)), the apparent K_d values are time-dependent (Fig. 2A). Expressing the same binding data as a function of the incubation time (Fig. 2B), reveals the relatively long delay for equilibrium binding to be attained at the lowest concentrations of [^{3}H]candesartan. Therefore, the K_d values given in Fig. 2 are only apparent. More elaborate kinetic experiments (Fig. 6) allowed the calculation of a pseudo first-order association rate constant (k_{obs}) of 0.113 ± 0.006 M^{-1} min^{-1} for 1.1 nM [^{3}H]candesartan.

Dissociation experiments were initially carried out by washing [^{3}H]candesartan-preincubated cells and subsequent incubation in fresh medium. Regardless of the initial [^{3}H]candesartan concentration, dissociation occurred very slowly, with a calculated first-order rate constant (k_{-1}) of 0.001 min^{-1} (Table 2, Fig. 3A). Isotopic dilution was used as an alternative method to investigate the dissociation of [^{3}H]candesartan. For this purpose, dissociation was initiated by (i) washing the [^{3}H]candesartan-pretreated cells and replacement with fresh medium containing an excess of unlabelled ligand (Fig. 3A) or (ii) by adding an excess

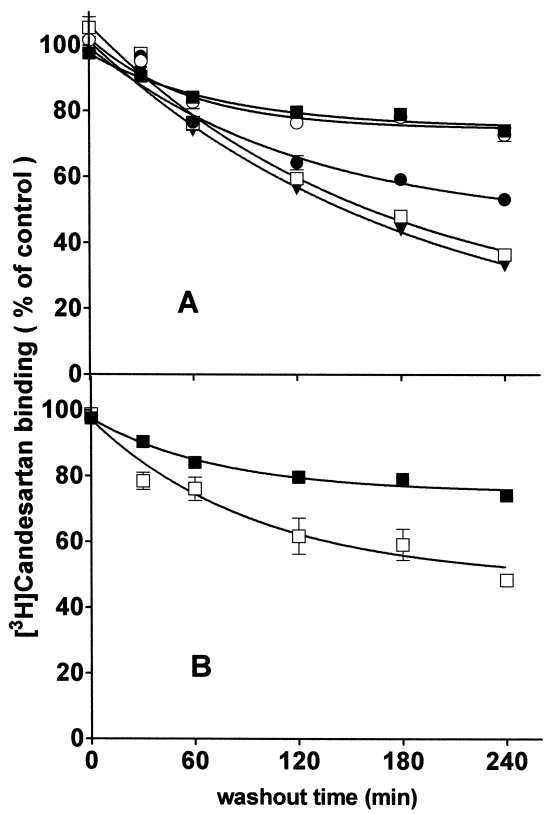

Fig. 4. Time course of [^{3}H]candesartan dissociation: Effect of losartan and bovine serum albumin. CHO-AT_1 cells were preincubated for 30 min with 1.1 nM [^{3}H]candesartan and dissociation of the radioligand was initiated by washing of the cells and replacement with fresh medium either alone (■), for Panel A, medium containing increasing concentrations of losartan (0.01 μM (○), 0.1 μM (●), 1.0 μM (□) or 10 μM (▼)), or for Panel B medium containing 0.1% Bovine serum albumin (□). Data are presented as in Fig. 3 and the k_{-1} values are given in Fig. 5 and Table 2.

of unlabelled ligand without removal of the free [^{3}H]candesartan (Fig. 3B). The dissociation rate constants were comparable for the two procedures and did not vary substantially when 0.1 μM candesartan, 0.1 μM EXP3174, 1 μM irbesartan, 10 μM losartan or 0.1 μM angiotensin II were used as ligand (Table 2). However, these dissociation rate constants were about 4 to 5-fold higher as the ones that were obtained by incubating the cells in fresh medium without ligand (Table 2). Thus, the dissociation of [^{3}H]candesartan is accelerated in the presence of unlabelled ligands. As shown in Fig. 4A (example for losartan) and Fig. 5, this acceleration was dose-dependent and the potency of the unlabelled ligands (candesartan > EXP3174 > irbesartan > losartan) was similar to the one previously found for occupying the angiotensin II AT_1 receptors in competition binding studies (Table 1). There was also an increase in the dissociation rate of [^{3}H]candesartan in the washout experiments when the fresh medium contained 0.1% Bovine serum albumin. The k_{-1} increased to 0.0025 ± 0.0005 min^{-1} (Table 2).

Two series of experiments were performed to investigate the relationship between [^{3}H]candesartan binding and its ability to inhibit the angiotensin II induced inositol phosphate accumulation. This response was measured by a 5-min incubation of the cells with a maximal effective concentration of angiotensin II (0.1 μM) (Vanderheyden et al., 1998). In the experiments shown in Fig. 6A, preincubation of the cells with 1.5 nM candesartan resulted in a time-dependent decrease of the angiotensin II induced response. This time-dependent decrease mirrored the association of 1.5 nM [^{3}H]candesartan to the angiotensin II AT_1 receptors. The washout experiments shown in Fig. 6B compare the dissociation of [^{3}H]candesartan with the recovery of the response. Both dissociation and recovery were allowed to proceed in washout medium containing 10 μM losartan. As outlined in Section 4, this prevents the reassociation of candesartan to its receptor sites and does not impair the subsequent stimulation of the receptors by a maximal effective concentration of angiotensin II. Under these conditions, there was a close match between the dissociation of [^{3}H]candesartan and the recovery of the angiotensin II induced inositol phosphate accumulation. Half-lives were 2.2 h and 2.6 h, respectively.

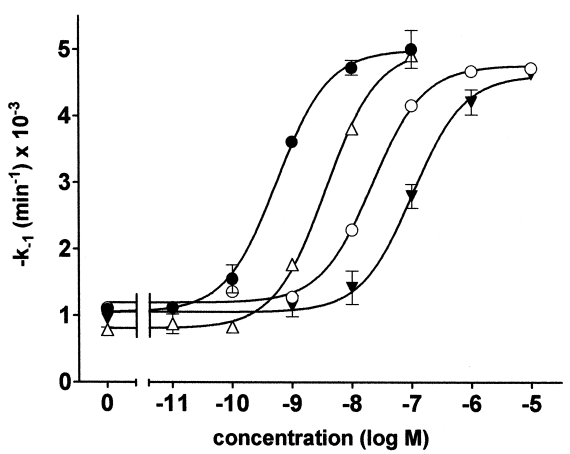

Fig. 5. Rate of [^{3}H]candesartan dissociation: effect of increasing concentrations of candesartan, EXP3174, irbesartan and losartan. Dissociation experiments in the presence of candesartan EXP3174 and irbesartan were performed as shown for irbesartan in Fig. 4. The calculated k_{-1} values are given for each concentration (abscissa) of candesartan (●), EXP3174 (Δ), irbesartan (♦) and losartan (▼). Half-maximal increase in the k_{-1} values was obtained for 0.6 ± 0.1 nM candesartan, 7.5 ± 1.3 nM angiotensin II, 5.7 ± 1.4 nM EXP3174, 23.6 ± 2.2 nM irbesartan and 105 ± 20 nM losartan.

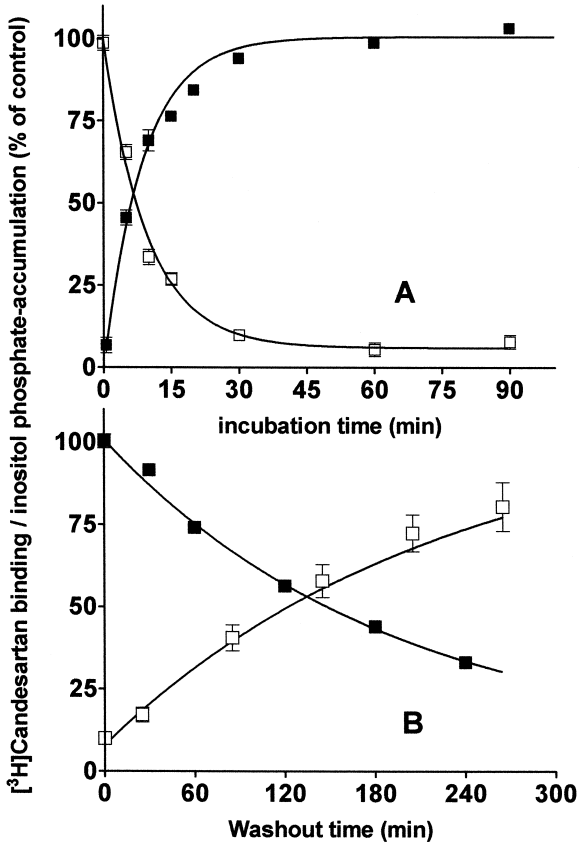

Fig. 6. Panel A: Time course of [^{3}H]candesartan association and the inhibitory effect of candesartan. Binding (■): CHO-AT_1 cells were incubated for increasing periods of time (abscissa) with 1.5 nM [^{3}H]candesartan, after which binding was measured. Angiotensin II induced Inositol phosphate responses (□): cells were preincubated for the indicated periods of time with 1.5 nM candesartan, and then further incubated with 0.1 μM angiotensin II for 5 min. Panel B: Time course of [^{3}H]candesartan dissociation and the inhibitory effect of candesartan. Binding (■): CHO-AT_1 cells were preincubated for 30 min with 1.5 nM [^{3}H]candesartan. Dissociation of the radioligand was initiated by washing of the cells and replacement with fresh medium containing 10 μM Losartan. Remaining binding was measured after incubation with medium for the indicated periods of time (abscissa). Angiotensin II induced Inositol phosphate responses (□): cells were subjected to the same treatment with candesartan 1.5 nM instead of radioligand, washed, further incubated with fresh supplemented medium containing 10 μM losartan, for the indicated periods of time and finally incubated with 0.1 μM angiotensin II for 5 min.

4. Discussion

The insurmountable behavior of the angiotensin II AT_1 receptor antagonist candesartan has initially been observed by measuring the contraction of rabbit aortic rings (Shibouta et al., 1993; Noda et al., 1995). When the aortic rings were exposed to candesartan prior to their challenge with angiotensin II, it produced an almost complete depression of the maximal contractile response. Recently, it was also shown that candesartan produces an insurmountable inhibition of the angiotensin II-mediated accumulation of inositol phosphates in CHO cells transfected with the gene coding for human angiotensin II AT_1 receptors (CHO-AT_1 cells) (Vanderheyden et al., 1998). In the present study, it is shown that [^{3}H]candesartan binds with high affinity and selectivity to the angiotensin II AT_1 receptors in CHO-AT_1 cells. The order of potency of unlabelled antagonists to displace the binding of [^{3}H]candesartan (candesartan > EXP3174 > irbesartan > losartan) is the same as for displacing the cell surface binding of [^{3}H]angiotensin II (Fig. 1, Table 1). The non-specific binding of [^{3}H]candesartan is negligible as compared with the total binding.

A crucial observation was that the response of candesartan-treated aortic rings and CHO-AT_1 cells to angiotensin II took very long to recover (Ojima et al., 1997; Vanderheyden et al., 1998). It has been suggested that this long-lasting inhibition implies that, immediately following the candesartan-treatment, angiotensin II is unable to stimulate all the available receptor molecules so that its dose-response curve becomes depressed. The insurmountable behavior of candesartan can therefore be directly linked to its ability to produce a long-lasting inhibition of the cell's responses.

An allosteric model has also been invoked to explain the interaction between angiotensin II AT_1 receptors and insurmountable antagonists (Timmermans et al., 1991). Such a modulation of ligand binding has already been shown to occur for several G protein-coupled receptors, including adrenoceptors and muscarinic, dopamine and adenosine receptors (Kostenis and Mohr, 1996). Experimentally, allosteric inhibition should involve a reduction in the association rate and/or an increase in the dissociation rate of the ligand but only the latter is conclusive (Kostenis and Mohr, 1996). Radioligand dissociation experiments are therefore frequently performed to find out if an inhibitor acts allosterically. In this respect, the dissociation of bound [^{3}H]candesartan was very slow when initiated by the incubation of the cells in fresh medium (k_{-1} 0.0010 ± 0.0001 min^{-1}) but it could be accelerated about 4 to 5-fold when this procedure was replaced by an isotopic dilution with unlabelled ligand and this irrespective of the prior removal of radioligand-containing medium (Figs. 3–5, Table 2). At first glance, this finding may be compatible with the existence of allosteric binding sites on the angiotensin II AT_1 receptor. However, it is difficult to conceive how different classes of ligands such as angiotensin II AT_1 receptor antagonists and even candesartan itself could increase the dissociation rate of [^{3}H]candesartan to the same degree (Table 2). According the hypothesis of allosteric binding sites, this latter finding is especially important since it should imply that candesartan interacts with its receptors in a negative cooperative fashion. Yet, the Hill coefficients of [^{3}H]candesartan saturation binding data collected so far are all close to unity: $n_H = 1$ for bovine adrenal cortical membranes (Ojima et al., 1997) and $n_H = 1.2 \pm 0.1$ for CHO-AT_1 cells (Fig. 2A).

The accelerated dissociation of [^{3}H]candesartan in the presence of unlabelled ligands could also reflect the capability of released radioligand molecules to re-associate to the angiotensin II AT_1 receptors. In this respect, receptor-saturating concentrations of any unlabelled ligand should prevent the re-association process. Accordingly, the dissociation rate constant determined by washing with buffer only is an underestimate (Table 2). The explanation of reassociation is supported by the fact that the dissociation of [^{3}H]candesartan is also accelerated in the presence of Bovine serum albumin. Bovine serum albumin is known to bind candesartan and concurrently, to reduce its free concentration as well as its potency in both binding and functional studies (Ojima et al., 1997; Vanderheyden et al., 1998). On the other hand, the very low non-specific binding of [^{3}H]candesartan in the present study does not support the proposal made by Panek et al. (1995) who suggested that the long-lasting action of certain angiotensin II type I antagonists in aortic strip contraction studies is related to slow removal from compartments within tissue, cells or matrix surrounding the angiotensin II AT_1 receptor, instead of slow dissociation from the receptor.

The re-association of [^{3}H]candesartan can easily be explained by the fact that the angiotensin II AT_1 receptor concentration (or bound radioligand) is not negligible in the present experimental set up. It amounts to 0.12 nM when the total incubation volume is taken into consideration. When only the cell volume is taken into consideration, the local concentration rises even to about 50 nM. This receptor concentration is much higher than the K_d for [^{3}H]candesartan. This constant ($K_d = 51 \pm 8$ pM) could not be directly determined by saturation binding experiments on CHO-AT_1 cells because of the long time required for equilibrium to be reached at low radioligand concentrations. Instead, it could be calculated from the association ($k_1 = 9.8 \pm 0.4 \times 10^7$ M^{-1} min^{-1}) and the accurate dissociation rate constant (i.e., in the absence of re-association) obtained by the kinetic studies. Computer-simulations based on these parameters (Fig. 7) reveal that, the 'apparent' k_{-1} value should decrease from 0.0050 min^{-1} to 0.0029 min^{-1} when the reassociation of released radioligand is included in the calculations. A further decline may occur when a local accumulation of released radioligand is also taken into consideration. The experimental k_{-1} value of 0.0010 min^{-1} (Fig. 5, Table 2) can be obtained when

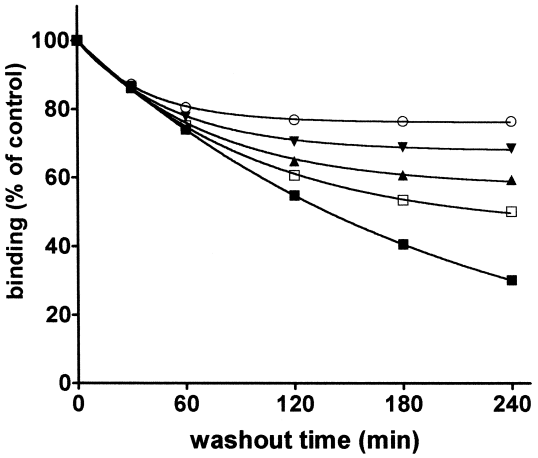

Fig. 7. Computer simulations and corresponding k_{-1} values for [^{3}H]candesartan dissociation according to different models; no re-association (■): 0.0050 min^{-1}; for re-association without accumulation (□, $n=1$): 0.0029 min^{-1}; for re-association with accumulation of released radioligand (▲, $n=2$): 0.0021 min^{-1}, (▼, $n=4$): 0.0015 min^{-1} and (●, $n=8$): 0.0009 min^{-1}. n refers to the factor of accumulation of the radioligand in the proximity of the receptor. The k_{-1} values are obtained by linear regression analysis of the ln(binding) vs. time representation with time points equal as in Fig. 5.

the concentration of free radioligand in the vicinity of the receptors is allowed to be 8-fold higher as compared to the situation in which it is homogeneously distributed in the medium.

Among the theories which have been proposed to explain the molecular basis for insurmountable antagonism, at least two of them do not necessitate the antagonist to remain bound to the receptor to produce a long-lasting effect. In the two-state model (Gero, 1983; Robertson et al., 1994), the receptor is able to adopt an active (R) an inactive (R') conformation and it is assumed (1) that insurmountable antagonists display higher affinity for the R' and (2) that the interconversion between the two receptor conformations is much slower than the ligand binding. Accordingly, the binding of insurmountable antagonists will result in a new equilibrium with less R and subsequently added agonists should be unable to bring the R/R' ratio back to normal during the relatively short time that is required for measuring the response. The maximal response remains therefore depressed. In the somewhat related coupling model of De Chaffoy de Courcelles et al. (1986), the crucial conformational change occurs at the cytoplasmic side of the receptor and it affects the coupling of a transducing factor which is involved in the generation of the cellular response.

The relevance of such mechanisms for candesartan can be evaluated by comparing its dissociation with the recovery of the angiotensin II-mediated accumulation of inositol phosphates in the CHO-AT$_1$ cells. These rates should obviously be determined when the re-association process is prevented. Whereas, the accurate dissociation rate of [^{3}H]candesartan can be measured in the presence of receptor-saturating concentrations of all types of unlabelled angiotensin II AT$_1$ receptor ligands, the recovery of the response can only be detected in the presence of rapidly dissociating (surmountable) antagonists such as losartan. Indeed, agonists have to be excluded because they produce receptor stimulation and desensitization on their own. Slow dissociating antagonists cannot be used either because of their own insurmountable behavior. Under these conditions, there is a close match between the recovery of the response of candesartan-treated CHO-AT$_1$ cells and the dissociation of bound [^{3}H]candesartan from these cells (Fig. 6). Hence, there is no necessity to invoke a delay between the dissociation of the antagonist and the restoration of the native receptor conformation (such as in the above models) to explain the insurmountable effect of candesartan. The antagonism by candesartan can therefore be directly linked to its ability to occupy the angiotensin II AT$_1$ receptors. The close match between the receptor occupation by [^{3}H]candesartan in association binding studies and the decline in the angiotensin II-mediated response is in full agreement with this proposition.

In agreement with the binding data, the angiotensin II response of candesartan-treated cells also recovered faster in the presence of losartan than in fresh medium only (Fig. 6 compared to Fig. 6 in Vanderheyden et al., 1998). Interestingly, a similar effect is also observed in aortic strip contraction studies (Robertson et al., 1992; Ojima et al., 1997) in which the effect of insurmountable angiotensin II AT$_1$ receptor antagonists such as GR117289 and candesartan is attenuated upon subsequent exposure of the strips to losartan. Because of the similarity with the present findings on CHO-AT$_1$ cells (Fig. 6B), it is likely that some re-association of insurmountable antagonists to the receptor therefore also takes place in aortic strip contraction experiments. In this context, the disposition studies by Kondo et al. (1996) pointed out that, following single or repeated p.o. administrations of [^{3}H]candesartan cilexetil to rats, there was a net accumulation of its active metabolite [^{3}H]candesartan in the blood vessels as well as in other tissues. The elimination of [^{3}H]candesartan from the blood vessels was slower than that from the plasma and this slow elimination was suggested to account for the sustained antihypertensive effect.

5. Conclusion

Binding studies with [^{3}H]candesartan reveal that this antagonist binds with high affinity and selectivity to the human angiotensin II AT$_1$ receptors in CHO-AT$_1$ cells. Candesartan depresses the maximal response of the cells (measured as inositol phosphates accumulation) to subsequently added angiotensin II. The present data indicate that

this insurmountable behavior of candesartan is related to its slow dissociation from the receptor. Our data indicate that the recovery of the angiotensin II mediated IP accumulation in CHO-AT_1 cells from candesartan treatment, the actual dissociation of the antagonist is required. Furthermore our data suggest the ability of candesartan to re-associate to the receptor and/or to accumulate in its vicinity during such experiments. Both phenomena result in the slow recovery of the responsiveness of candesartan-treated CHO-AT_1 cells to angiotensin II (Vanderheyden et al., 1998). It is likely that these phenomena are also relevant in other experimental systems, such as in rabbit aortic strips, but this remains to be firmly established.

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References

Balmforth, A.J., Lee, A.L., Warburton, P., Donnelly, D., Ball, S.G., 1997. The conformational change responsible for AT_1 receptor activation is dependent upon two juxtaposed asparagine residues on transmembrane heIices III and VII. J. Biol. Chem. 272, 4245–4251.

Bumpus, F.M., Catt, K.J., Chiu, A.T., DeGasparo, M., Goodfriend, T., Husain, A., Peach, M.J., Taylor, D.G. Jr., Timmermans, P.B., 1991. Nomenclature for angiotensin receptors. A report of the Nomenclature Committee of the Council for High Blood Pressure Research. Hypertension 17, 720–721.

Cazaubon, C., Gougat, J., Bousquet, F., Guiraudou, P., Gayraud, R., Lacour, C., Roccon, A., Galindo, G., Barthelemy, G., Gautret, B., Bernhart, C., Perreaut, P., Breliere, J.-C., Le Fur, G., Nisato, D., 1993. Pharmacological characterization of SR 47436, a new nonpeptide AT_1 subtype angiotensin II receptor antagonist. J. Pharmacol. Exp. Ther. 265, 826–834.

Chang, R.S., Siegl, P.K., Clineschmidt, B.V., Mantlo, N.B., Chakravarty, P.K., Greenlee, W.J., Patchett, A.A., Lotti, V.J., 1992. In vitro pharmacology of L-158,809, a new highly potent and selective angiotensin II receptor antagonist. J. Pharmacol. Exp. Ther. 262, 133–138.

De Chaffoy de Courcelles, D., Leysen, J.E., Roevens, P., Van Belle, H., 1986. The serotonin-S2 receptor: a receptor, transducer coupling model to explain insurmountable antagonist effects. Drug Dev. Res. 8, 173–178.

Dickinson, K.E., Cohen, R.B., Skwish, S., Delaney, C.L., Serafino, R.P., Poss, M.A., Gu, Z., Ryono, D.E., Moreland, S., Powell, J.R., 1994. BMS-180560, an insurmountable inhibitor of angiotensin II-stimulated responses: comparison with losartan and EXP3174. Br. J. Pharmacol. 113, 179–189.

Gero, A., 1983. Desensitization, two-state receptors and pharmacological parameters. J. Theor. Biol. 103, 137–161.

Iwai, N., Inagami, T., 1992. Identification of two subtypes in the rat type I angiotensin II. FEBS Lett. 298, 257–260.

Kakar, S.S., Sellers, J.C., Devor, D.C., Musgrove, L.C., Neill, J.D., 1992. Angiotensin II type-1 receptor subtype cDNAs: differential tissue expression and hormonal regulation. Biochem. Biophys. Res. Commun. 183, 1090–1096.

Kondo, T., Yoshida, K., Yoshimura, Y., Motohashi, M., Tanayama, S., 1996. Disposition of the new angiotensin II receptor antagonist candesartan cilexetil in rats and dogs. Arzneim.-Forsch./Drug Res. 46, 594–600.

Kostenis, E., Mohr, K., 1996. Two-point kinetic experiments to quantify allosteric effects on radioligand dissociation. Trends Pharmacol. Sci. 17, 280–283.

Liu, Y.J., Shankley, N.P., Welsh, N.J., Black, J.W., 1992. Evidence that the apparent complexity of receptor antagonism by angiotensin II analogues is due to a reversible and synoptic action. Br. J. Pharmacol. 106, 233–241.

Mochizuki, S., Sato, T., Furata, K., Hase, K., Ohkura, Y., Fukai, C., Kosakai, K., Wakabayashi, S., Tomiyama, A., 1995. Pharmacological properties of KT3-671, a novel nonpeptide angiotensin II receptor antagonist. J. Cardiovasc. Pharmacol. 25, 22–29.

Noda, K., Saad, Y., Karnik, S.S., 1995. Interaction of Phe8 of angiotensin II with Lys199 and His256 of ATl receptor in agonist activation. J. Biol. Chem. 270, 28511–28514.

Ojima, M., Inada, Y., Shibouta, Y., Wada, T., Sanada, T., Kubo, K., Nishikawa, K., 1997. Candesartan (CV-11974) dissociates slowly from the angiotensin AT_1 receptor. Eur. J. Pharmacol. 319, 137–146.

Panek, R.L., Lu, G.H., Overhisser, R.W., Major, T.C., Hodges, J.C., Taylor, D.G., 1995. Functional studies but not receptor binding can distinguish surmountable from insurmountable AT1 antagonism. J. Pharmacol. Exp. Ther. 273, 753–761.

Perlman, S., Schamby, H.T., Rivero, R.A., Greenlee, W.J., Hjorth, S.A., Schwartz, T.W., 1995. Non-peptide angiotensin agonist. Functional and molecular interaction with the ATl receptor. J. Biol. Chem. 270, 1493–1496.

Robertson, M.J., Barnes, J.C., Drew, G.M., Clark, K.L., Marshall, F.H., Michel, A., Middlemiss, D., Ross, B.C., Scopes, D., Dowle, M.D., 1992. Pharmacological profile of GR 117289 in vitro: a novel, potent and specific non-peptide angiotensin AT_1 receptor antagonist. Br. J. Pharmacol. 107, 1173–1180.

Robertson, M.J., Dougall, I.G., Harper, D., Mckechnie, K.C.W., Leff, P., 1994. Agonist-antagonist interactions at angiotensin receptors: application of a two-state receptor model. Trends Pharmacol. Sci. 15, 364–369.

Seeuwen, K., Lagarde, A., Pouysségur, J., 1988. Deregulation of hamster fibroblast proliferation by mutated ras oncogenes is not mediated by constitutive activation of phosphoinositide-specific phospholipase C. EMBO J. 7, 161–168.

Shibouta, Y., Inada, Y., Ojima, M., Wada, T., Noda, M., Sanada, T., Kubo, K., Kohara, Y., Naka, T., Nishikawa, K., 1993. Pharmacological profile of a highly potent and long-acting angiotensin II receptor antagonist, 2-ethoxy-l-[[2.45-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid (CV-11974), and its prodrug, (±)-1-(cyclohexyloxy-carbonyloxy)-ethyl2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biohenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate (TCV-116). J. Pharmacol. Exp. Ther. 266, 114–120.

Timmermans, P.B.M.W.M., Wong, P.C., Chiu, A.T., Herblin, W.F., 1991. Nonpeptide angiotensin II receptor antagonists. Trends Pharmacol. Sci. 12, 55–62.

Timmermans, P.B.M.W.M., Benfield, P., Chiu, A.T., Herblin, W.F., Wong, P.C., 1992. Angiotensin II receptors and functional correlates. Am. J. Hypertens. 5, 221S–235S.

Vallotton, M.B., 1987. The renin–angiotensin system. Trends. Pharmacol., Sci. 8, 69–74.

Vanderheyden, P.M.L., Fierens F.L.P., De Backer, J.-P., Frayman, N., Vauquelin, G., 1998. Distinction between surmountable and insurmountable selective AT_1 receptor antagonists by use of CHO-K1 cells expressing human angiotensin II AT_1 receptors. Br. J. Pharmacol. (in press).

Wong, P.C., Price, A.W., Chiu, A.T., Duncia, J.V., Carini, D.J., Wexler, R., Johnson, A., Timmermans, P.B.M.W.M., 1990. Nonpepride angiotensin receptor antagonists. XI. Pharmacology of EXP3174: An active metabolite of DuP 753, an orally active antihypertensice agent. J. Pharmacol. Exp. Ther. 255, 211–217.

Zhang, J.C., Van Meel, A., Pfaffendorf, M., Van Zwieten, P., 1993. Different types of angiotensin II receptor antagonism induced by BIBS 222 in the rat portal vein and rabbit aorta; the influence of receptor reserve. J. Pharmacol. Exp. Ther. 269, 509–514.