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Antagonist interaction with endogenous AT_1 receptors in human cell lines

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Abstract

Using Chinese Hamster Ovary cells expressing human AT_1 receptors cells (CHO-hAT_1), it was previously shown that insurmountable inhibition of the angiotensin II response by non-peptide antagonists is related to the duration of their receptor occupancy. In the present study it was shown that these antagonists displayed similar binding characteristics to endogenously expressed AT_1 receptors in human adrenal cortex cells (NCI-h295) and renal vascular smooth muscle cells (HVSMC). Competition binding studies with [^{3}H]candesartan for NCI-h295 cells, with [^{125}I]Sar^1–Ile^8 angiotensin II for HVSMC and with both radioligands for CHO-hAT_1 cells displayed the same potency order for unlabelled antagonists: candesartan > EXP3174 > irbesartan > losartan. The AT_2 receptor antagonist PD123319 displayed low potency in all instances. The apparent half-lives of the antagonist–AT_1 receptor complexes in NCI-h295 cells and HVSMC were comparable to those obtained under identical conditions with CHO-hAT_1 cells. Angiotensin II increased the inositol phosphate accumulation dose dependently with half-maximal response at 17.4 ± 1.6 nM for NCI-h295 cells and 4.5 ± 0.8 nM for HVSMC. Pre-incubation of the cells with losartan only produced concentration-dependent rightward shifts of the angiotensin II concentration–response curve. The maximal response was decreased by 85–92% with candesartan, 70–88% with EXP3174 and 60% with irbesartan. The similar binding and inhibitory properties of these antagonists among the investigated cell types validates the use of CHO-hAT_1 cells for investigating pharmacological properties of human AT_1 receptors.

Keywords: Angiotensin II; Human AT_1 receptor; Vascular smooth muscle; NCI-h295; Antagonist; Insurmountable

1. Introduction

Angiotensin II is the most important peptide in the renin–angiotensin system. It exerts a considerable role in the regulation of blood pressure and sodium homeostasis and most of its actions, including contraction of vascular smooth muscle cells and aldosterone secretion by adrenal glomerulosa cells, are mediated by the AT_1 receptor [1]. Selective non-peptide AT_1 receptor antagonists have been developed for the clinical treatment of hypertension. These antagonists are traditionally studied by measuring their ability to influence angiotensin II dose–response curves of rabbit aorta strips, a system with small receptor reserve [2]. They are divided in two categories, based on their inhibitory action. Those, which only produce a parallel rightward shift of the dose–response curve, are noted as surmountable while those, which cause an additional depression of the maximal response, are insurmountable. The extent by which these antagonists depress the maximal response ranges from partial (i.e. irbesartan, valsartan and EXP3174) to almost complete (candesartan) [3–5]. Slow dissociation kinetics from the AT_1 receptor underlie insurmountable antagonism [6] and the extended occupancy of the AT_1 receptor by certain antagonists has been proposed by some to lengthen the duration of their antihypertensive effect [7]. Compared to contraction studies with rabbit aortic strips, cell lines expressing the endogenous or transfected AT_1 receptors offer major advantages for the investigation of antagonists [8]. In particular, their pharmacological properties can be

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Abbreviations: AT_1, Angiotensin II receptor type 1; BSA, bovine serum albumin; CHO-hAT_1 cells, Chinese Hamster Ovary cells expressing human AT_1 receptors; DMEM, Dulbecco's Modified Eagle's Medium; HVSMC, human renal vascular smooth muscle cells; NCI-h295, human adrenal cortex cells; PBS, phosphate-buffered saline.

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investigated both by radioligand binding and by functional experiments under the same experimental conditions. To this end, CHO-hAT$_1$ cells were previously used to compare the interaction of this receptor with the non-peptide antagonists losartan, valsartan, irbesartan, EXP3174 and candesartan. When pre-incubated with the CHO-hAT$_1$ cells, these antagonists affected angiotensin II concentration–inositol phosphate production–response curves in a similar fashion as in contractile studies. It was found that these antagonists were reversible and competitive inhibitors and that the insurmountable inhibition in antagonist pre-incubation experiments was related to their ability to form tight binding/slow reversible complexes with the AT$_1$ receptor [8,9]. Compared with the transfected CHO-hAT$_1$ cells, cells derived from human kidney arterial blood vessels (HVSMC) and NCI-h295 express AT$_1$ receptors in an endogenous fashion and at a lower, physiologically more relevant density. In the present study, we compared the effects of AT$_1$ receptor antagonists on these human cells by binding studies with [^{125}I]Sar^1–Ile^8 angiotensin II and [^{3}H]candesartan and by measuring their effect on the angiotensin II-mediated inositol phosphate production.

The accumulation of lipophilic antagonists in the vicinity of the receptor has been advanced as an explanation for their long-lasting/insurmountable inhibition [10]. Therefore, receptor-independent accumulation of [^{3}H]candesartan and [^{3}H]irbesartan was also checked for.

2. Materials and methods

2.1. Materials

Candesartan (CV-11974; 2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid), EXP3174 (2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid), losartan (DuP 753; 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole) and irbesartan (SR 47436; 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]2-imidazolin-5-one) and the tritiated ligands [^{3}H]candesartan (22 Ci/mmol) and [^{3}H]irbesartan (5 Ci/mmol) were obtained from AstraZeneca, [^{125}I]Sar^1–Ile^8 angiotensin II (2200 Ci/mmol) from Perkin–Elmer. Angiotensin II was obtained from Neosystem. The AT$_2$ receptor antagonist PD123319 and BSA were obtained from Sigma. *myo*-[^{3}H]Inositol (20 Ci/mmol) was obtained from Amersham Biosciences. All other chemicals were of the highest grade commercially available.

2.2. Cell culture

CHO-hAT$_1$ cells were obtained and cultured as described [8]. HVSMC were obtained from a primary culture [11]. Cells were cultured in 75-cm^2 flasks in Dulbecco's Modified Essential Medium (DMEM) which was supplemented with L-glutamin (2 mM), 2% of a stock solution containing 5000 IU/mL penicillin and 5000 μg/mL streptomycin, 1% (v/v) of a solution of MEM containing non-essential amino acids, 1 mM sodium pyruvate and 10% (v/v) fetal calf serum (Invitrogen). Experiments were performed with cells grown to confluence in 12-well plates (inositol phosphate accumulation) or 24-well plates ([^{125}I]Sar^1–Ile^8 angiotensin II binding studies).

NCI-h295 cells were obtained from the American Type Culture Collection, cultured in 75-cm^2 flasks in DMEM/Dulbecco's Modified Essential Ham's F-12 Medium which was supplemented with insulin (6 μg/mL), transferrin (3.3 μg/mL) and selenium (4 ng/mL) (ITS solution, Invitrogen), 2.5% Ultroser (Invitrogen), lineolic acid (4 μg/mL)–BSA (1 mg/mL) (Sigma–Aldrich) and 2% of a stock solution containing 5000 IU/mL penicillin and 5000 μg/mL streptomycin (Invitrogen). Experiments were performed with cells grown to confluence in 12-well plates ([^{3}H]candesartan binding studies) or 24-well plates (inositol phosphate accumulation).

2.3. [^{125}I]Sar^1–Ile^8 angiotensin II binding

Before the experiment, cells (CHO-hAT$_1$ or HVSMC) were washed twice with HEPES-buffered DMEM (0.5 mL per well) at room temperature. After removal of the medium, 225 μL HEPES-buffered DMEM + 0.1% BSA was added to each well and the plate was then left for 15 min at 37°.

All further incubations were performed in this medium. Competition binding experiments were performed by pre-incubating the cells for 30 min at 37° with increasing concentrations of unlabelled antagonists and further incubation with antagonist plus 0.05 nM [^{125}I]Sar^1–Ile^8 angiotensin II for 60 min in a final volume of 250 μL.

For association kinetics, cells were incubated for the indicated time periods at 37° with 0.05 nM [^{125}I]Sar^1–Ile^8 angiotensin II. The dissociation rate constants of unlabelled antagonists were determined by measuring their ability to delay the association of [^{125}I]Sar^1–Ile^8 angiotensin II as earlier described [12]. For this purpose, cells were pre-incubated for 30 min at 37° with 250 μL medium containing unlabelled candesartan (10 nM), EXP3174 (10 nM) or irbesartan (100 nM). Cells were briefly washed twice with PBS containing 0.9 mM $CaCl_2{\cdot}2H_2O$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.49 mM $MgCl_2{\cdot}6H_2O$, 137 mM NaCl and 8 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.4) at 4°, and incubated at 37° for the indicated time periods with 250 μL medium containing 0.05 nM [^{125}I]Sar^1–Ile^8 angiotensin II. The concentrations of the unlabelled antagonists were chosen to obtain full receptor occupancy, as verified by competition binding with [^{125}I]Sar^1–Ile^8 angiotensin II. At the end of the experiments, the plates were placed on ice and the cells were washed three times with PBS at 4°, treated overnight with 400 μL of a 1 mol/L sodium hydro-

xide at room temperature. The radioactivity of the solution was counted in a γ-counter (Perkin–Elmer). Non-specific [^{125}I]Sar^1–Ile^8 angiotensin II binding, measured in the presence of 1 μM unlabelled candesartan, was subtracted from the total binding to yield specific binding. Binding parameters from the association binding curves (k_{obs}) and competition binding curves (log [IC_{50}]) were calculated by non-linear regression analysis using GraphPad Prism. Data points were the mean ± SEM of at least three separate experiments with triplicate determinations each. The apparent dissociation rate constants of the unlabelled antagonists were calculated by computer-assisted iteration according to a model of competitive interaction [12].

In parallel with the binding assays the protein concentration was determined as described previously [12]: they were 0.218 ± 0.012 and 0.036 ± 0.004 mg/mL for CHO-hAT_1 cells and HVSMC, respectively.

2.4. [^{3}H]Candesartan binding

Before the experiment, NCI-h295 cells were washed twice with DMEM (0.8 mL per well) at room temperature. After removal of the medium, 0.9 mL DMEM was added and the plate was then left for 15 min at 37°.

For saturation binding assays cells were incubated with increasing concentrations of [^{3}H]candesartan (final concentrations between 0.05 and 5 nM) in a final volume of 1 mL. Competition binding experiments were performed by pre-incubating the cells for 30 min at 37° with increasing concentrations of unlabelled antagonists and further incubation with antagonist plus 1.5 nM [^{3}H]candesartan for 30 min in a final volume of 1 mL.

For association kinetics, cells were incubated for the indicated periods of time at 37° with 1.5 nM [^{3}H]candesartan. For the [^{3}H]candesartan dissociation experiments, cells were incubated with 1.5 nM radioligand for 30 min at 37° in a final volume of 1 mL. The dissociation of the radioligand was initiated by addition of 1 μM unlabelled candesartan or by washing of the cells and subsequent exposure to medium either without or containing 1 μM candesartan. The cells were then further incubated for the indicated time periods. At the end of each incubation, the cells were briefly washed three times with PBS at 4°. The cell-bound radioactivity in each well was solubilised with 500 μL sodium hydroxide (0.2 mol/L) and counted for 3 min in a liquid scintillation counter after addition of 3 mL scintillation liquid (Optisafe, Perkin–Elmer). Non-specific binding, measured in the presence of 1 μM unlabelled candesartan, was subtracted from the total binding to yield specific [^{3}H]candesartan binding. The calculation of the binding parameters from the dissociation curves (k_{-1}), association curves (k_{obs}), saturation binding curves (K_D and B_{max} values) and competition curves (K_i values) was performed by non-linear regression analysis using GraphPad Prism. Data points were the mean ± SEM of at least three separate experiments with triplicate determinations each. The protein concentration of the NCI-h295 cells in the binding assay was 0.087 ± 0.006 mg/mL.

2.5. Inositol phosphate accumulation

The cells were plated in 24-well plates and cultured near to confluence. The medium was replaced by supplemented DMEM containing 1 μCi/mL *myo*-[^{3}H]inositol and the cells were further grown for 20 hr in 5% CO_2 at 37° until confluence. To investigate the effect of AT_1 receptors antagonists on angiotensin II concentration–response curves, the cells were first washed two times with DMEM and left in 400 μL DMEM containing 10 mM LiCl for 15 min at 37°. Pre-incubations were initiated by addition of 50 μL medium without (controls) or with the indicated concentrations of AT_1 receptor antagonist and proceeded at 37° for 30 min. Subsequent incubations were initiated by adding 50 μL medium without (basal accumulation) or with increasing concentrations angiotensin II and proceeded at 37 ° for 5 min.

The inositol phosphate accumulation represented the measurement of mono-, bi- and trisphosphates as described by Vanderheyden *et al*. [8]. For the concentration–effect curves, the responses were given as percentage of the maximal angiotensin II response (E_{max}) in the absence of antagonist pre-treatment. All values were means ± SEM of at least three experiments with triplicate determinations each.

2.6. Accumulation experiments

For indicated time periods, cells were incubated with 3 and 50 nM [^{3}H]candesartan or [^{3}H]irbesartan. At corresponding time points (30, 60 and 90 min), non-specific binding in the presence of 10 μmol/L unlabelled angiotensin II was measured. At least three separate experiments with triplicate determinations each were performed.

3. Results

3.1. [^{125}I]Sar^1–Ile^8 angiotensin II binding to HVSMC and CHO-hAT_1 cells

The AT_1 receptor expression in HVSMC was too low to allow binding studies with tritiated ligands but accurate measurements could be performed with the radioiodinated peptide antagonist [^{125}I]Sar^1–Ile^8 angiotensin II. Specific binding of 0.05 nM [^{125}I]Sar^1–Ile^8 angiotensin II to intact HVSMC and CHO-hAT_1 cells is 3.37 ± 0.36 and 119.9 ± 4.1 fmol/mg protein, respectively. On HVSMC, specific binding of 0.05 nM [^{125}I]Sar^1–Ile^8 angiotensin increases time-wise with a k_{obs} of 0.0117 ± 0.0006 min^{-1}(N = 3, Fig. 1). The pseudo first-order association rate constant obtained by corresponding experiments on CHO-hAT_1 cells is 0.0149 ± 0.0016 min^{-1} (N = 3, Fig. 1).

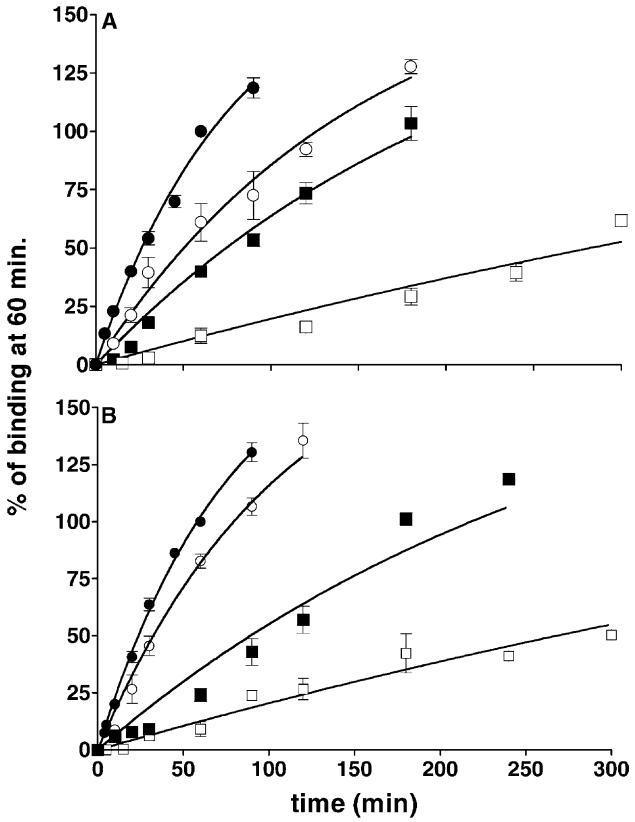

Fig. 1. Effect of antagonist pre-incubation on the association rate of [^{125}I]Sar^1–Ile^8 angiotensin II. (A): CHO-hAT$_1$ cells, (B): HVSMC cells. Data are the average ± SEM of three experiments performed in triplicate and are expressed as percentage of control binding at 60 min (20276 ± 699 cpm for (A), 2470 ± 283 cpm for (B). Pre-incubation with medium only ((●) control), (○) irbesartan, (■) EXP3174 and (□) candesartan.

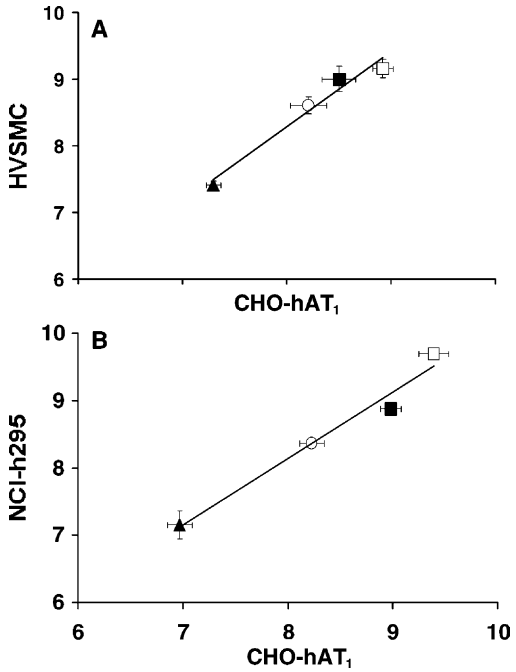

Fig. 2. Correlation of log [IC$_{50}$] values of competition experiments with CHO-hAT$_1$ cells and HVSMC ((A): ligand [^{125}I]Sar^1–Ile^8 angiotensin II) and NCI-h295 cells, ((B): ligand [^{3}H]candesartan). (▲) Losartan, (○) irbesartan, (■) EXP3174 and (□) candesartan. The r^2 values are given in the text.

The dissociation rate constants ($k_{-1(I)}$) of candesartan and other non-peptide AT$_1$ receptor antagonists were estimated indirectly. Pre-incubation of HVSMC and CHO-hAT$_1$ cells with the unlabelled antagonists delay the association of subsequently added [^{125}I]Sar^1–Ile^8 angiotensin II. For both cell lines, this delay is most pronounced for candesartan, intermediate for EXP3174 and the smallest for irbesartan (Fig. 1). Assuming that this delay reflects the dissociation of the unlabelled antagonists from the receptors, their apparent k_{-1} values can be calculated by computer-assisted iteration [12]. Values of three separate experiments are 0.0056 ±0.0007 min^{-1} (candesartan), 0.022±0.004 min^{-1} (EXP3174) and 0.031 ± 0.010 min^{-1} (irbesartan) for CHO-hAT$_1$ cells and 0.0064 ± 0.0015 min^{-1} (candesartan), 0.016 ± 0.003 min^{-1} (EXP3174) and 0.068 ± 0.020 min^{-1} (irbesartan) for HVSMC.

[^{125}I]Sar^1–Ile^8 angiotensin II is able to bind AT$_1$ as well as AT$_2$ receptors but up to 10 μmol/L of AT$_2$ receptor-selective antagonist PD123319 fails to displace its binding to HVSMC and CHO-hAT$_1$ cells. The tested AT$_1$ receptor ligands produce a concentration-dependent and complete inhibition of the specific binding of this radioligand to HVSMC and CHO-hAT$_1$ cells. The potency order is candesartan > EXP3174 > irbesartan > losartan for and there is a good correlation between the potency ratios of the different antagonists for both cell lines ($r^2 = 0.96$) when comparing log [IC$_{50}$] values (Fig. 2A, N = 3).

3.2. [^{3}H]Candesartan binding to NCI-h295 cells

Specific binding of 1.5 nM [^{3}H]candesartan to NCI-295 cells increases time dependently until a plateau value is reached (data not shown). The corresponding pseudo first-order association rate constant (k_{obs}) is 0.18 ± 0.02 min (N = 3). The dissociation rate of the radioligand was determined by different methods. Cells were first incubated with 1.5 nM [^{3}H]candesartan for 30 min and then either:

a. left in the presence of 1 μM candesartan (isotopic dilution) for increasing periods of time at 37°, or
b. washed and further incubated with fresh medium containing 1 μM candesartan, or
c. washed and further incubated with fresh medium alone.

In all instances, binding decreases exponentially with time. When the potential association/rebinding of free [^{3}H]candesartan to the receptor is prevented by the presence of a receptor saturating concentration of candesartan (1 μM), the calculated k_{-1} values are 0.0052 ± 0.0005 min^{-1} (a) and 0.0064 ± 0.0006 min^{-1} (b). When rebinding of dissociated radioligand molecules can still take place (c), binding decreases at a slower pace with an apparent k_{-1}

value of $0.0033 \pm 0.0002\ min^{-1}$. These values are SEM of three separate experiments. Analysis with an unpaired *t*-test established that when rebinding of free [^{3}H]candesartan is prevented, k_{-1} values are significantly different ($P < 0.05$) from the value obtained when rebinding can still take place. When calculated from the kinetic data in the absence of rebinding, the equilibrium dissociation constant of [^{3}H]candesartan (K_D) is 45 pM (a) and 54 pM (b).

The specific binding of [^{3}H]candesartan to the NCI-295 cells is saturable. Yet, an appreciably higher K_D value (0.18 ± 0.02 nM, N = 3) is obtained by non-linear regression analysis of the saturation binding data as compared to the values calculated from the kinetic data. The same discrepancy has earlier been noticed for CHO-hAT$_1$ cells [13]. Because of the long time required for equilibrium to be reached at low radioligand concentrations, it was attributed to an overestimation of the "thermodynamic" K_D value. The AT$_1$ receptor concentration on the NCI-295 cells, corresponding to the B_{max} values of the saturation binding experiments, is 620 ± 70 fmol/mg protein (SEM from three experiments).

Competition binding experiments with [^{3}H]candesartan reveal that all the tested AT$_1$ receptor ligands produce a concentration-dependent and complete inhibition of the specific binding. The potency order of the AT$_1$ antagonists is candesartan > EXP3174 > irbesartan > losartan. There is a good correlation between the potency ratios of the different antagonists for both cell lines ($r^2 = 0.97$) when comparing log [IC$_{50}$] values for displacing [^{3}H]candesartan binding to NCI-295 cells and to CHO-hAT$_1$ cells [13], (Fig. 2B, N = 3). The AT$_2$ receptor-selective antagonist PD123319 did not displace the binding of [^{3}H]candesartan to the NCI-295 cells at concentrations up to 10 μmol/L.

3.3. Inositol phosphate accumulation

In the presence of 10 mM LiCl, angiotensin II causes a concentration-dependent accumulation of inositol phosphate in HVSMC and NCI-h295 cells with an EC$_{50}$ of 4.5 ± 0.8 and 17.4 ± 1.6 nM, respectively (Fig. 3, N = 3). Maximal stimulation is obtained with 0.1 μmol/L angiotensin II and is about four times the basal inositol phosphate level. A 30-min pre-incubation of both cell lines with increasing concentrations of the AT$_1$ receptor antagonists candesartan, EXP3174 and irbesartan causes a biphasic reduction in the maximal angiotensin II-mediated inositol phosphate accumulation. Curves in Fig. 4 show, as an example, the effect of irbesartan and candesartan on NCI-h295 cells (N = 3). The most potent component of these curves corresponds to insurmountable inhibition [9] and comprises approximately 91% (for candesartan), 88% (for EXP3174) and 60% (for irbesartan) of the maximal response in HVSMC and 85% (for candesartan), 70% (for EXP3174) and 60% (for irbesartan) of the maximal response NCI-h295 cells. Fig. 3 shows the effect of single

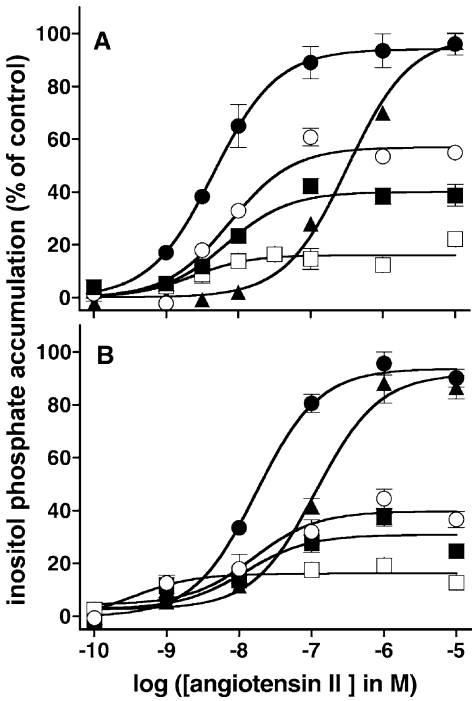

Fig. 3. Concentration–response curves of angiotensin II-mediated inositol phosphate accumulation in (A) HVSMC and (B) NCI-h295 cells after 30-min pre-incubation with medium only ((●) control), (□) 5 nM candesartan, (■) 5 nM EXP3174, (○) 10 nM irbesartan and (▲) 100 nM losartan for NCI-h295 cells and 1000 nM losartan for HVSMC. Data are expressed as in Fig. 1 (632 ± 84 cpm for (A) and 651 ± 22 cpm for (B).

antagonist concentrations (at which maximal insurmountable inhibition is achieved) on the angiotensin II dose–response curve for both cell lines (N = 3). In contrast, pre-incubation of both cell lines with losartan only produces

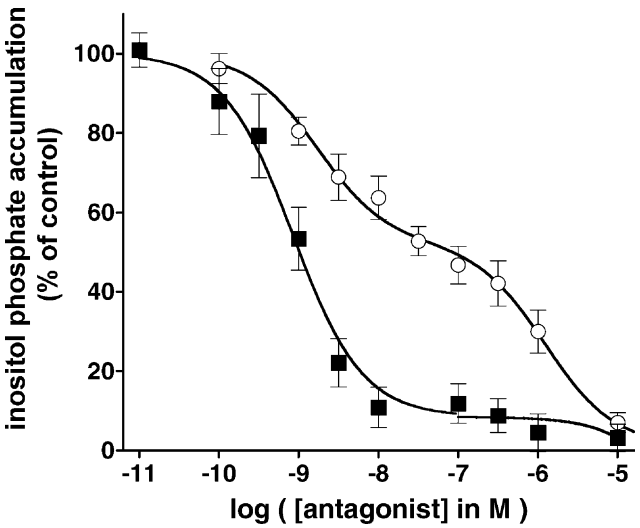

Fig. 4. Inhibition curves of angiotensin II-mediated inositol phosphate accumulation of (○) irbesartan and (■) candesartan. NCI-h295 cells are pre-incubated for 30 min with the indicated concentrations of the antagonists (abscissa) and then incubated for 5 min with 10 μmol/L angiotensin II after which the amount of accumulated inosiol phosphates is determined. Data are the average ± SEM of three experiments, performed in duplicates or triplicates, and expressed as percentage of the maximal angiotensin II response (1271 ± 124 cpm).

a rightward shift of the concentration–response curve without affecting the maximal response.

3.4. Non-receptor [^{3}H]candesartan and [^{3}H]irbesartan binding

To compare potential receptor-independent accumulation of [^{3}H]candesartan and [^{3}H]irbesartan, non-specific binding of both radioligands to HVSMC, NCI-h295 and CHO-hAT$_1$ cells was measured in the presence of a AT$_1$ receptor-masking concentration (10 μmol/L) of angiotensin II. When binding is expressed as a function of total cell protein content, non-receptor binding for 3 nM [^{3}H]candesartan and [^{3}H]irbesartan on the three cell lines is not significantly different from the counter background. At 50 nM, non-specific binding is below 0.1% of total radioactivity added and remains constant for a 30-, 60- and 90-min incubation (data not shown).

4. Discussion

The present study reveals that the binding properties of non-peptide antagonists to AT$_1$ receptors in intact HVSMC and NCI-h295 cells are very similar to those earlier described for the transfected CHO-hAT$_1$ cells.

Competition experiments reveal that the antagonists display the same potency and potency order (candesartan > EXP3174 > irbesartan > losartan) for displacing the binding of [^{3}H]candesartan to NCI-h295 and CHO-hAT$_1$ cells (Fig. 2B). The same potency and potency order is also found when comparing competition binding data with [^{125}I]Sar^1–Ile^8 angiotensin II on HVSMC and CHO-hAT$_1$ cells (Fig. 2A). Earlier competition studies on CHO-hAT$_1$ cells with [^{3}H]irbesartan, [^{3}H]valsartan and [^{3}H]angiotensin II yielded the same potency order [13–15].

Earlier experiments shed light on the marked differences in the dissociation rate of the different antagonists from the AT$_1$ receptors on CHO-hAT$_1$ cells [12]. They comprised dissociation experiments with radiolabelled antagonists as well as indirect determinations based on the delayed recovery of the angiotensin II-mediated inositol phosphate accumulation and the delayed association of [^{3}H]candesartan in wash-out experiments with antagonist pre-treated cells. In this respect, it has been alluded that (radio)ligands that dissociated from a receptor will accumulate in the medium and, provided that they have a very high affinity for the receptor, that they may undergo ‘‘reassociation’’ or ‘‘rebinding’’ to the same or a nearby receptor on the cell surface [16,17]. This phenomenon can effectively be prevented by the radioligand in delayed association experiments and by the presence of unlabelled ligand in radioligand dissociation experiments [12].

Here, the delayed association of [^{125}I]Sar^1–Ile^8 angiotensin II was introduced to calculate the antagonist dissociation half-lives. Calculated values for candesartan (108 min), EXP3174 (44 min) and irbesartan (10 min) for the HVSMC are similar to the values obtained with the same radioligand for CHO-hAT$_1$ cells (123, 31 and 22 min, respectively) as well as to those earlier obtained for CHO-hAT$_1$ cells (152, 31 and 17 min, respectively) based on the delayed association of [^{3}H]candesartan [12]. These indirect values for candesartan are also similar to those obtained by directly measuring the dissociation of [^{3}H]candesartan in the presence of an excess of losartan from the CHO-hAT$_1$ cells (138 min) and, in the present study, from the NCI-h295 cells (108 to 133 min). On the other hand, when the [^{3}H]candesartan dissociation experiments are performed by simply replacing the radioligand containing medium with fresh medium, the dissociation is significantly delayed both for the CHO-hAT$_1$ [13] and the NCI-h295 cells. Since this delay is likely to result from the rebinding of released radioligand, the corresponding kinetic data may not reflect the true dissociation rate of the radioligand.

Taken together, the present competition binding data and the kinetic data indicate that the binding properties of the tested non-peptide AT$_1$ receptor antagonists remain the same whether the human AT$_1$ receptors are transfected and expressed in a foreign cellular environment (CHO-hAT$_1$ cells) or whether they are endogenously expressed (HVSMC and NCI-h295 cells). In addition, as the AT$_1$ receptors are expressed at appreciably higher levels in CHO-hAT$_1$ cells as in the human cell lines, the antagonist binding properties do not appear to be influenced by the receptor concentration either.

The AT$_1$ receptor antagonists show also marked differences in their ability to depress the maximal effects of angiotensin II. This depression (or ‘‘insurmountable antagonism’’) necessitates the pre-exposure of the AT$_1$ receptors with the antagonist before their challenge with angiotensin II, and can be observed in vascular smooth muscle contraction studies as well as by determining the accumulation of inositol phosphates in CHO-hAT$_1$ cells. After 30-min pre-incubation of these cells with various antagonists, the maximum angiotensin II-induced inositol phosphate accumulation was depressed by about 95% for candesartan, 70% for EXP3174 and 30% for irbesartan. No such depression was observed for losartan [8]. A similar inhibitory behaviour of these antagonists has also been observed in contraction studies with rabbit aortic strips and rat portal vein [3,5]. These phenomena are routinely demonstrated with angiotensin II concentration–response curves (Fig. 3) but they become even more outspoken when portraying the experimental data under the form of inhibition curves (Fig. 4) [9]. In these curves, the most potent component corresponds to insurmountable inhibition (independent of the angiotensin II concentration) and the least potent component to surmountable inhibition (dependent of the angiotensin II concentration). The presence of a distinct plateau between both components facilitates the determination of the maximal extend of

insurmountable inhibition by each antagonist. The fact that the maximal response was only partially depressed by each of the insurmountable antagonists was explained by a two inactive-state competition [9] where:

1. antagonist–AT_1 receptor complexes may adopt two distinct states: a fast reversible state which accounts for the surmountable inhibition by the antagonist and a tight binding state which accounts for the insurmountable inhibition by the antagonist;
2. an equilibrium between both states can be attained: this equilibrium depends on the structure of the antagonist.

In this study, it was found that candesartan, EXP3174, irbesartan and losartan depressed the angiotensin II-induced inositol phosphates accumulation in HVSMC and NCI-h295 cells to the same degree as in earlier studies on CHO-hAT_1 cells. This similarity indicates that the density and cellular environment of the human AT_1 receptors have little effect on their ability to adopt fast and slowly reversible complexes with each antagonist.

Similar to [^{3}H]valsartan [15], [^{3}H]candesartan binding displays only one-phase dissociation and competition curves in this and previous studies [13]. It is likely that the surmountable antagonist–receptor complexes dissociated during washing procedure at the end of the incubation so that measured [^{3}H]candesartan binding only reflects the insurmountable receptor complex.

Among the alternative theories that have been advanced to explain the insurmountable behaviour of AT_1 receptor antagonists in contraction studies, Panek *et al*. [10] raised the issue of slow antagonist removal from tissue compartments, cells or matrix surrounding the AT_1 receptor. This could be governed by factors such as lipid solubility, receptor environment and kinetics of distribution and metabolism of the antagonist within the tissue [10]. In the same line, Robertson *et al*. [18] noticed that certain insurmountable antagonists, such as GR117289, are highly lipophilic and it was suggested that such ligands could be retained or, perhaps, even be concentrated in the membrane lipid. In the present study, no evidence could be found for such lipophilicity-related accumulation of AT_1 receptor antagonists. Indeed, although irbesartan is appreciably more hydrophobic than candesartan, the non-specific (non-receptor) binding of both tritiated antagonists was excessively small and quite similar for each cell line. These data suggest that the insurmountable/long-lasting action of AT_1 receptor antagonists is more likely to be related to their slow dissociation from the receptor than to their slow removal from the immediate surroundings of the receptor.

Interestingly, a moderate but consistent difference became apparent when comparing the EC_{50} of the angiotensin II-stimulated inositol phosphates accumulation in NCI-h295 cells (17.4 ± 1.6 nM) as compared to CHO-hAT_1 cells (3.4 ± 0.7 nM) and HVSMC (4.5 ± 0.8 nM). The occurrence of a non-linear stimulus–response function or ''receptor reserve'' in the latter cell lines as compared to a linear stimulus–response function in the NCI-h295 cells constitutes the simplest interpretation for this discrepancy. Yet, previous kinetic experiments comparing the maximal angiotensin II-mediated inositol phosphates production with the cell surface binding of [^{3}H]angiotensin II and [^{3}H]candesartan [8,13] plead against the occurrence of such AT_1 receptor reserve in CHO-hAT_1 cells. At the present level of investigation, one can only speculate about the alternative causes for the lower EC_{50} of the angiotensin II-stimulated inositol phosphates accumulation in NCI-h295 cells and, in this respect, differences in receptor microenvironment and/or in its G-protein coupling offer an appealing explanation.

In conclusion, antagonist–human AT_1 receptor interactions were indistinguishable, whether this receptor is expressed in the foreign Chinese Hamster Ovary cell system or endogenously expressed in HVSMC or NCI-h295. These interactions appear, therefore, to be insensitive to the receptor concentration as well as to species-related differences in the G-proteins or other cellular components with which the receptors may potentially interact. The similar binding and inhibitory properties of these antagonists among the investigated cell types validate the use of CHO-hAT_1 cells for investigating pharmacological properties of human AT_1 receptors.

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