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Ligand binding and functional properties of human angiotensin AT_1 receptors in transiently and stably expressed CHO-K1 cells

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Abstract

Chinese Hamster Ovary Cells (CHO-K1) were transiently and stably transfected to express the human angiotensin AT_1 receptor. Cell surface receptor expression was maximal 2 days after transient transfection. Their pharmacological and signalling properties differed from stably expressed receptors. Receptor reserve was significant in the transient cells but not in stable cells, explaining the higher potency of angiotensin II and the lower degree of insurmountable inhibition by candesartan in the transient cells. [Sar^1Ile^8]angiotensin II (sarile) is a potent angiotensin AT_1 receptor antagonist for the stable cells but is a partial agonist, producing 19% of the maximal response by angiotensin II, in transient cells. Internalization of [^{3}H]angiotensin II and [^{125}I]sarile (i.e., acid-resistant binding) was more pronounced in stable cells. CHO-K1 cells were also transiently transfected with the enhanced green fluorescence-AT_1 receptor gene. Confocal microscopy revealed rapid internalization induced by angiotensin II and sarile but not by candesartan. The above disparities may result from differences in receptor maturation and/or cellular surrounding.

Keywords: CHO-K1; Angiotensin II; Angiotensin AT_1 receptor; Internalization; Receptor reserve

1. Introduction

The octapeptide hormone angiotensin II exerts most of its physiological effects by stimulating angiotensin AT_1 receptors. Starting with the activation of Gq, the angiotensin AT_1 receptors elicit an increased production of inositol phosphate and a transient rise in cytoplasmic calcium concentration. By its combined effects on cardiovascular, endocrine and neuronal systems, angiotensin II is one of the most potent regulators of blood pressure and it is an important etiological factor in hypertension and cardiovascular disease (De Gasparo et al., 2000). Two strategies have been highly successful in counteracting these pathologies. Angiotensin converting enzyme inhibitors have been introduced to decrease the plasma level of angiotensin II and angiotensin AT_1 receptor antagonists have been developed to selectively block the access of angiotensin II to this receptor (Timmermans, 1999; Unger, 1999).

Initial attempts to block the actions of angiotensin II relied on the development of synthetic analogues of this peptide. These were obtained by substituting one or more of the original amino acids of angiotensin II by natural or synthetic amino acids. However, such peptide analogues often display partial agonistic activity and this constituted one of the reasons for the failure of the therapeutic use of saralasin ([Sar^1,Val^5,Ala^8]angiotensin II) (Pals et al., 1979). [Sar^1,Ile^8]angiotensin II (sarile) is another typical example and, in its radioiodinated form, it is widely used for the labelling of angiotensin AT_1 as well as angiotensin AT_2 receptors in radioligand binding studies. Like angiotensin II,

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it displays high affinity (i.e., K_d in the nanomolar range) for both receptor subtypes. Based on its ability to inhibit angiotensin II-mediated angiotensin AT_1 receptor stimulation, sarile appears to behave as a potent angiotensin AT_1 receptor antagonist. In the same line 'in vitro' contraction studies of rabbit aorta and rat portal vein revealed that sarile produced a significant, concentration-dependent depression of the maximum contractile force mediated by angiotensin II (Pendleton et al., 1989; Wienen et al., 1992; Zhang et al., 1994). This depression, referred to as "insurmountable inhibition," occurred when the vascular tissue was exposed to sarile before its challenge with angiotensin II. Therefore, it attributed to the long-lasting action of this antagonist (Pendleton et al., 1989; Wienen et al., 1992). In agreement with its insurmountable inhibition, the binding of [^{125}I]sarile to angiotensin AT_1 receptor containing membranes (Wienen et al., 1992) and cells (Le et al., 2003) has been shown to be slowly reversible.

More recently, a fair number of nonpeptide angiotensin AT_1 receptor-selective antagonists have been developed and some of those belonging to the biphenyltetrazole group have been highly successful in clinical practice. In vascular smooth muscle contraction studies, some of these antagonists also act in an insurmountable, long-lasting fashion while others only produce parallel rightward shifts of the angiotensin II concentration–response curve (i.e., "surmountable inhibition"). "In vitro" studies on recombinant systems such as CHO cells stably expressing the human angiotensin AT_1 receptor (CHO-hAT_1 cells) as well as on cell lines which express endogenous angiotensin AT_1 receptors (Verheijen et al., 2003) improved our understanding about the molecular action mechanism of these antagonists. In these cells, all investigated biphenyltetrazole antagonists were competitive with angiotensin II (Vanderheyden et al., 2000) but they displayed marked differences with respect to their ability to adopt fast reversible/surmountable and tight binding/insurmountable complexes with the angiotensin AT_1 receptor (Vauquelin et al., 2001a). The behaviour of these antagonists in CHO-hAT_1 cells and other cell lines with endogenous angiotensin AT_1 receptors is remarkably similar to that in more complex experimental systems like vascular smooth muscle preparations. Whereas losartan, the prototype of the nonpeptide antagonists, is only able to form fast reversible complexes, other antagonists like candesartan almost exclusively form tight binding complexes. In accordance with the formation of tight-binding complexes, [^{3}H]candesartan was found to bind with high affinity to intact CHO-hAT_1 cells and to dissociate slowly from these cells (Fierens et al., 1999a,b; Vauquelin et al., 2001a).

When comparing different studies, it emerges that the interaction of angiotensin AT_1 receptors with peptide and nonpeptide ligands could be affected by its mode of expression. Whereas candesartan pre-incubation produced a more than 90% inhibition of the maximal angiotensin II-stimulated inositol phosphate accumulation in CHO-hAT_1 cells and human cell lines with endogenous angiotensin AT_1 receptors (Fierens et al., 1999b; Verheijen et al., 2002), the inhibition did not exceed 80% in CHO-K1 cells that were transient expressing human angiotensin AT_1 receptors (Le et al., 2003). In the same vein, while sarile completely inhibited the angiotensin II receptor-mediated calcium influx in bovine adrenal glomerulosa cells (Ambroz and Catt, 1992), this peptide behaved as a partial agonist for recombinant cell lines transiently expressing angiotensin AT_1 receptors (Noda et al., 1995; Hines et al., 2001; Miserey-Lenkei et al., 2001; Le et al., 2003). These discrepancies prompted us to perform a comparative characterisation of the binding and functional properties of sarile and candesartan in CHO cells stably and transiently expressing human angiotensin AT_1 receptors. Differences in the inhibitory characteristics of candesartan in functional studies appeared to be dictated by the extent of "receptor reserve" in each cell system. On top of this, differences in the functional and binding characteristics of sarile between both cell systems were also observed.

2. Materials and methods

2.1. Materials

2-Ethoxy-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benzimidazoline-7-carboxylic acid (candesartan) and [^{3}H]candesartan (17 Ci/mmol) were obtained from AstraZeneca (Mölndal, Sweden). [^{3}H]angiotensin II (68 Ci/mmol) was obtained from Amersham, Biosciences (The Netherlands). Angiotensin II and [Sar^{1}Ile^8]angiotensin II (sarile) were obtained from Neosystem (France). LipofectAMINE was from Invitrogen (Belgium). [^{125}I]-[Sar^{1}Ile^8]angiotensin II (2200 Ci/mmol) was from Perkin Elmer Life Science (USA). Monoclonal anti-HA mouse IgG antibodies and FITC-labeled sheep antimouse IgG antibodies were from Sigma. All other chemicals were of the highest grade commercially available.

2.2. Cell culture and transient transfection

CHO cells stably expressing the human angiotensin AT_1 receptor were obtained and cultured as described (Vanderheyden et al., 1999). The cells were cultured in 12- or 24-well plates in DMEM (Dulbecco's modified essential medium) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 I.U./ml penicillin, 100 μg/ml streptomycin, 1% solution of non-essential amino acids, and 1 mM sodium pyruvate. Cells were grown in 5% CO_2 at 37 °C. To obtain transient expression, the human angiotensin AT_1 receptor gene was transfected into CHO-K1 cells at 80% confluence. Transfection was performed in Opti-MEM I by replacing the culture medium with the mixture of LipofectAMINE (8 μl/ml) and plasmid DNA (1.5 μg/ml). The cells were then incubated for 5 h and the supplemented DMEM

was added to terminate the transfection. After transfection, the cells were cultured from 1 to 4 days for the experiments shown in Fig. 1 and for 2 days for all other experiments.

2.3. Measurement of inositol phosphate accumulation

CHO cells were labelled by overnight incubation in complete DMEM containing 1 μCi/ml myo-[^{3}H]inositol (Amersham, Biosciences). After washing and pre-incubation with 10 mM LiCl for 15 min, 10 μM angiotensin II was added for another 15-min incubation. To investigate the effect of antagonists, the cells were pre-incubated with indicated concentrations of candesartan or sarile for 30 min before starting the incubation with angiotensin II. At the end of the incubation, the medium was removed and [^{3}H]inositol phosphate was extracted and measured as described (Vanderheyden et al., 1999).

2.4. Radioligand binding assays

Binding experiments were carried out on intact adherent cells cultured in 24-well plates as described (Le et al., 2003). Briefly the cells were incubated at 37 °C with [^{3}H]angiotensin II or [^{3}H]candesartan in DMEM or with [^{125}I]sarile in Hepes–DMEM containing 0.1% bovine serum albumine for all binding experiments. Saturation binding was performed for 60 min with 0.3 to 20 nM [^{3}H]angiotensin II or with 0.05 to 5 nM [^{125}I]sarile or [^{3}H]candesartan. Kinetic experiments (association and dissociation) were performed with 0.5 nM [^{125}I]sarile or with 1.5 nM [^{3}H]candesartan. Non-specific binding was measured in the presence of 1 μM unlabelled candesartan. Total cell binding: at the end of the incubation, the cells were washed three times with Krebs–Ringer buffer at 4 °C, solubilized with 0.2 M NaOH (0.5 ml/well), and the cell-bound radioactivity was measured in a liquid scintillation counter (for [^{3}H]angiotensin II and [^{3}H]candesartan) or in a gamma counter (for [^{125}I]sarile). To extract the acid-sensitive and acid-resistant binding of [^{3}H]angiotensin II and [^{125}I]sarile: at the end of the incubation, the cells were washed three times with ice-cold Krebs–Ringer buffer and then incubated for 5 min with 0.5 ml ice-cold acetic acid in 150 mM NaCl, pH 2.5. The radioactivity in the medium was measured to quantitate acid-sensitive binding. The remaining cells were solubilized with 0.2 M NaOH (0.5 ml/well) and 0.25% SDS (sodium dodecyl sulphate) and the radioactivity was measured to quantitate acid-resistant binding.

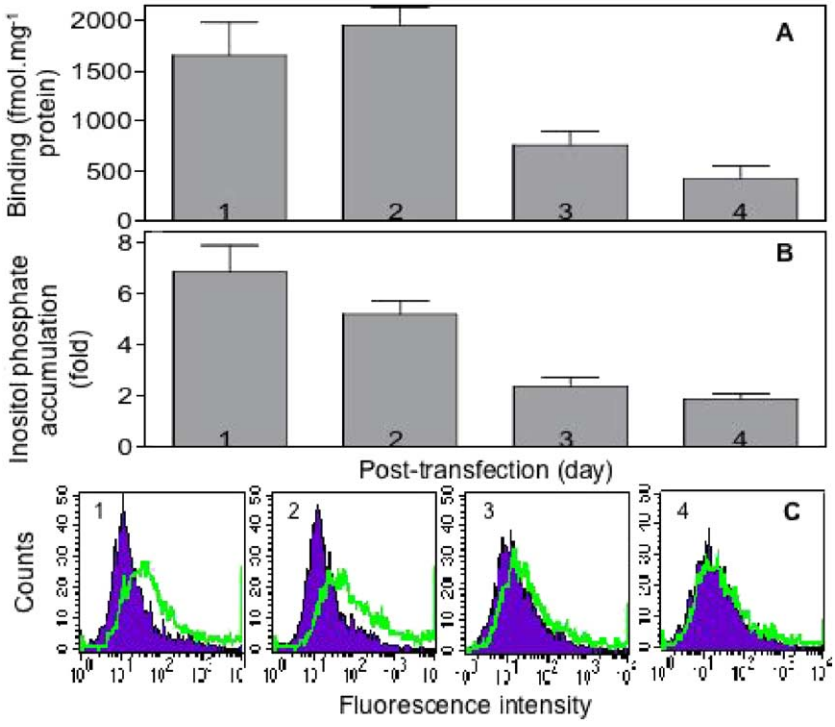

Fig. 1. Post-transfection analysis. CHO-K1 cells were transfected with HA epitope-tagged human angiotensin AT_1 receptor gene and transient expression assays were performed at days 1 to 4 of post-transfection as indicated in numbers. (A) Cells were incubated with 5 nM [^{3}H]candesartan at 37 °C for 60 min, and the binding was calculated by subtracting non-specific binding from total binding and was represented as fmole per mg protein. (B) Measurement of inositol phosphate accumulation after the cells were incubated with 10 μM angiotensin II at 37 °C for 15 min. Maximal stimulation is produced at 1 and 2 days post-transfection and corresponding to a 6- and 5-fold increase over the basal inositol phosphate level, respectively. (C) FACS analysis of 1 million cells using 5 μg anti-HA monoclonal antibody and 4 μg fluorescence-labelled secondary antibody. Distribution of fluorescence intensity of the cells was highest at days 1 and 2. The background signal was obtained in the absence of the monoclonal antibody and indicated by shaded peaks. There was no statistical difference ($P > 0.05$) between the 1 and 2 days post-transfection as assessed by a Mann–Whitney test.

2.5. Fluorescent detection of HA epitope-tagged angiotensin AT_1 receptors

CHO-K1 cells were transfected with the wild-type HA (hemagglutinin influenza) epitope-tagged human angiotensin AT_1 receptor gene and cultured at 37 °C for 1 to 4 days. 10^6 Cells were resuspended in 100 μl PBS (phosphate-buffered saline) and incubated with 5 μg of monoclonal anti-HA mouse IgG antibodies (Sigma) at 4 °C for 45 min. Cells were washed two times with PBS containing 0.002% Triton X-100 and 0.01% sodium azide, and then incubated with 4 μg of FITC-labeled sheep antimouse IgG (Sigma) at 4 °C for 30 min. After three washes with PBS, cells were resuspended in 2 ml PBS and analysed with a FACS (Fluorescent Activated Cell Shorter) flow cytometer (Becton Dickinson). Cells incubated with FITC-labeled sheep antimouse IgG only were used as background.

2.6. Confocal analysis of the angiotensin AT_1 receptor in CHO-K1 cells

CHO-K1 cells were plated onto 13-mm diameter glass coverslips inside 12-well plates until 80% confluence. The cells were transfected with 0.75 μg/ml EGFP-AT_1 receptor cDNA (Enhanced Green Fluorescent Protein-fused angiotensin AT_1 receptor) using 8 μl/ml LipofectAMINE. Two days after transfection, the cells were washed with DMEM and incubated with 0.1 μM angiotensin II, 0.1 μM sarile or 0.01 μM candesartan at 37 °C for 15 min. The internalization was stopped by ice-cold PBS washing and the cells were fixed with 2% formaldehyde at room temperature for 20 min. The coverslips were placed onto slide, mounted with DAPI mounting medium and examined in an inverted confocal microscope under a 40× oil immersion objective. GFP fluorescence was visualized using 488-nm laser line.

2.7. Data analysis

The binding and functional parameters were calculated by non-linear regression analysis using Graph Pad Prism (San Diego, CA, USA). K_d values were calculated according to the equation: $K_d = k_{-1}$ [L]/($k_{obs} - k_{-1}$). The method described by Umland et al. (2001) was applied to estimate the degree of apparent receptor reserve in the inositol phosphate accumulation experiments. Equipotent angiotensin II concentrations for untreated and candesartan-pretreated cells (i.e., $[A]$ and $[A']$, respectively) were calculated and the equilibrium dissociation constant of angiotensin II (i.e., K_A) was calculated from the linear $1/[A]$ versus $1/[A']$ plot by the equation: K_A=(slope-1)/y-intercept. For the untreated cells, the fractional receptor occupancy (i.e., ρ) at each agonist concentration was calculated as $\rho = ([A]/([A]+K_A)) \times 100$. Data points were the mean ± SEM of at least three separate experiments with duplicate or triplicate determinations.

3. Results

3.1. Transient expression of the human angiotensin AT_1 receptor in CHO-K1 cells

CHO-K1 cells were transiently transfected with the gene coding for the human angiotensin AT_1 receptor using LipofectAMINE (see Materials and methods section). Binding of a saturating concentration of the nonpeptide antagonist [^{3}H]candesartan (5 nM) to intact cells was evaluated at 1 to 4 days after the transfection. As shown in Fig. 1A, the highest levels of specific binding were detected at 2 days post-transfection and a gradual decline took place thereafter. In the same line, the angiotensin II-stimulated inositol phosphate accumulation was observed 1 to 2 days post-transfection. At a maximally effective concentration of angiotensin II (10 μM), this stimulation resulted in a ±6-fold increase over the basal inositol phosphate level (Fig. 1B).

The AT_1 receptor expression level was also assessed by flow cytometry. To this end, N-terminal HA tagged angiotensin AT_1 receptors at the cell surface were detected using a HA-binding monoclonal antibody. As for the binding and functional studies, the immunoreactivity was most outspoken 1 to 2 days post-transfection and it was almost completely disappeared after 4 days (Fig. 1C). Based on these results, binding and functional properties of the angiotensin AT_1 receptor in transiently transfected CHO-K1 cells (further denoted as transient cells) were characterised 2 days post-transfection. These properties were compared with those of the stably expressed angiotensin AT_1 receptors in the CHO-hAT_1 cell line (further denoted as stable cells).

3.2. Interaction with the nonpeptide antagonist candesartan

Angiotensin II elicited a concentration-dependent increase of the inositol phosphate accumulation with an EC_{50} of 0.92 ± 0.17 nM for transient cells and of 2.08 ± 0.11 nM for stable cells (Fig. 2A). Pre-incubation of the cells with 1 nM candesartan for 30 min reduced the maximal response to subsequently added angiotensin II by 55% for the transient cells and by 80% for the stable cells (Fig. 2A). The effect of candesartan was further investigated by pre-incubating the cells with increasing concentrations of candesartan followed by an incubation with a fixed, maximally effective concentration of angiotensin II (10 μM). The resulting inhibition curves were biphasic for both cell systems (Fig. 2B). As established previously, this reflects the presence of both tight binding/insurmountable and loose binding/surmountable candesartan–receptor complexes. When compared to the stable cells, the candesartan concentration–inhibition curve was slightly shifted to the right and the proportion of insurmountable inhibition was lower for the transient cells (75 ± 0.3% versus 85 ± 2.0%, Fig. 2B). Earlier experiments with stable cells revealed that

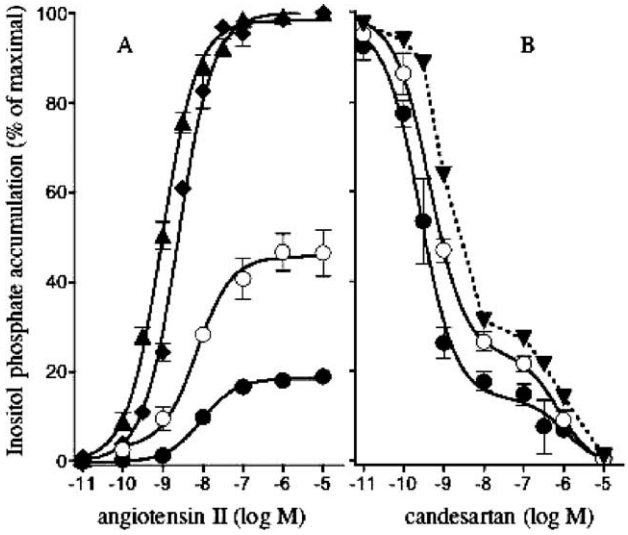

Fig. 2. Effect of candesartan pre-incubation on the angiotensin II induced inositol phosphate accumulation on both transient and stable cells. Panel (A) shows concentration–response curve of angiotensin II after 15 min incubation with increasing angiotensin II concentration on transient expressed cells (▲) and stable (♦) cells. Pre-incubation of 1 nM candesartan (30 min at 37 °C) decreased the maximal angiotensin II response on both transient (○) and stable (●) cells. Panel (B) shows candesartan concentration–inhibition curves of angiotensin II-mediated inositol phosphate accumulation on transient (○) and stable (●) cells. Cells were pre-incubated for 30 min at 37 °C with increasing concentration of candesartan, and then further incubated with 10 μM of angiotensin II. The dotted line represents the simulated curve of candesartan for stable cells in the assumption that they contain the receptor reserve.

this proportion is closely related to the dissociation rate of the investigated antagonist (Vauquelin et al., 2001b). However, when comparing the association and dissociation rates of 1.5 nM [^{3}H]candersartan for both cell systems, no substantial difference could be observed (Fig. 3, rate constants are given in Table 1).

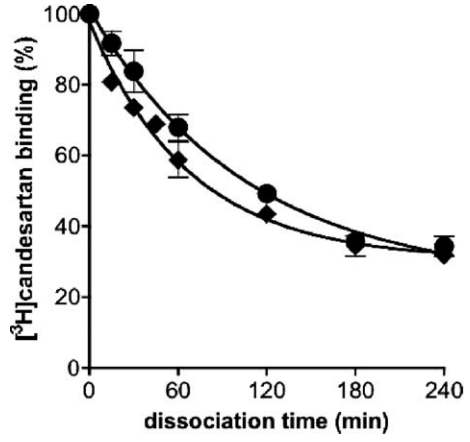

Fig. 3. Dissociation of [^{3}H]candesartan binding on the angiotensin AT_1 receptor transiently (♦) and stably (●) expressed CHO-K1 cells. Cells were incubated with 1.5 nM [^{3}H]candesartan in DMEM at 37 °C for 60 min. Dissociation of [^{3}H]candesartan was measured by the addition of 1 μM unlabelled candesartan. Values were given as percentage of maximal specific binding.

Table 1
Kinetic binding of [^{3}H]candesartan on CHO-K1 cells transient and stable transfected with human angiotensin AT_1 receptor

Parameters	Transient expression	Stable expression
k_{obs} (M^{-1} min^{-1})	0.101 ± 0.005	0.187 ± 0.012
k_{-1} (min^{-1})	0.015 ± 0.002	0.010 ± 0.001
K_d (nM)	0.262 ± 0.048	0.085 ± 0.014
B_{max} (fmol mg^{-1} protein)	479 ± 44	705 ± 10

k_{obs} and k_{-1} values were obtained from the association and dissociation curves of [^{3}H]candesartan. B_{max} and K_d values were derived from saturation curves by non-linear regression analysis using GraphPad Prism (San Diego, USA). All values represent mean ± SEM of at least 3 independent experiments.

3.3. Receptor reserve analysis

The linearity of the stimulus–response relationship was assessed in both cell systems according to the method described by Umland et al. (2001). This method is based on the comparison of equiactive agonist concentrations in systems with the initial and with artificially reduced receptor numbers. Because of its very slow dissociation, candesartan was used to reduce the angiotensin AT_1 receptor concentration in the present study. Following a 60-min pre-treatment of the cells with candesartan, free antagonist molecules were removed by washing with DMEM at 37 °C and cells were then immediately incubated with increasing concentrations of angiotensin II. Under these conditions, the maximal angiotensin II-induced inositol phosphate accumulation was reduced by at least 50% with 0.5 nM candesartan for the stable cells and with 1.2 nM of the antagonist for the transient cells. Based on the reciprocal values of equiactive angiotensin II concentrations in untreated and candesartan-treated cells (Fig. 4. inserts), the K_A value of angiotensin II was calculated to be 1.5 nM for stable cells and 2.9 nM for transient cells. Fig. 4 compares the receptor occupancy by angiotensin II (based on the K_A values) versus its capacity to induce inositol phosphate accumulation in both cells. Stable cells showed no evidence for angiotensin AT_1 receptor reserve: Angiotensin II generated 50% of the maximal response at 49.9% receptor occupancy (Fig. 4A). On the other hand, in transient cells, only 23% of the receptors needed to be occupied to generate 50% of the maximal response (Fig. 4B). Non-linear regression of the occupancy versus response curve for the transient cells yielded the following hyperbolic equation (with response and receptor occupancy expressed as percent of maximal):

$$\text{Response} = 135/(1 + (37/\text{receptor occupancy})) \quad (1)$$

Simulation studies based on this equation (data not shown) reveal that the non-linear stimulus–response relationship in the transient cells is sufficient to explain the difference in potency of angiotensin II in both cell systems (Fig. 2A). The same mechanism also adequately explains

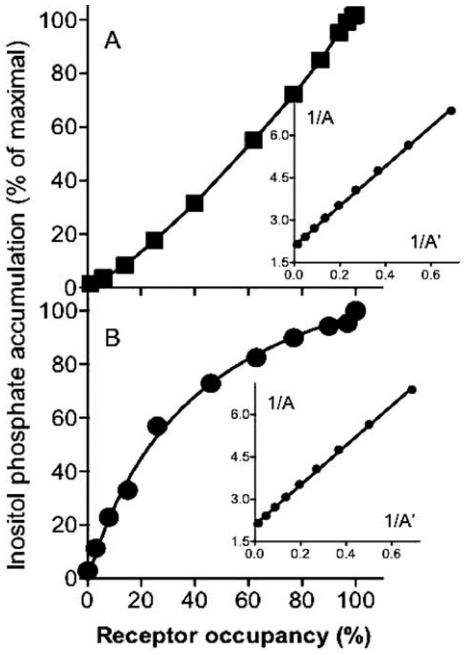

Fig. 4. Receptor reserve analysis in the functional assays. A comparison of receptor occupancy versus angiotensin II-induced inositol phosphate response in CHO cells stably (A) or transiently (B) expressed the human angiotensin AT_1 receptor. The corresponding insert figures show a linear plot of equiactive concentrations of angiotensin II with and without candesartan. The calculated K_A values were 1.5 nM (slope=2.1 and y-intercept=0.69) for stable cells and 2.9 nM (slope=7.1 and y-intercept=2.1) for transient cells.

the lower proportion of insurmountable inhibition by candesartan in the transient cells (Fig. 2B).

3.4. Interaction with [Sar^{1}Ile^8]angiotensin II (sarile)

Similar as for candesartan (Fig. 2B), biphasic inhibition curves were generated when transient and stable cells were pre-incubated with increasing concentrations of sarile and then further incubated with 10 μM angiotensin II (Fig. 5A). However, the high affinity component of the inhibition curve was appreciably less outspoken for the transient cells as for the stable ones. Only part of this difference could be explained by the non-linear stimulus–response relationship in the transient cells. Indeed, transformation of the angiotensin II-mediated responses for the stable cells according to Eq. (1) reduced the magnitude of the high affinity component of the sarile inhibition curve (Fig. 5A, dotted line for the simulated curve) but this reduction was still insufficient for a fit with the experimental data for the transient cells. This unforeseen complexity prompted us to check for the ability of sarile to produce a response on its own in both cell systems. Whereas this peptide produced no measurable response in the stable cells, it produced a concentration-dependent increase in the inositol phosphate accumulation in the transient cells with an EC_{50} of about 2 nM (Fig. 5B). In these latter cells, the maximal response of sarile was 19% of the one generated by angiotensin II.

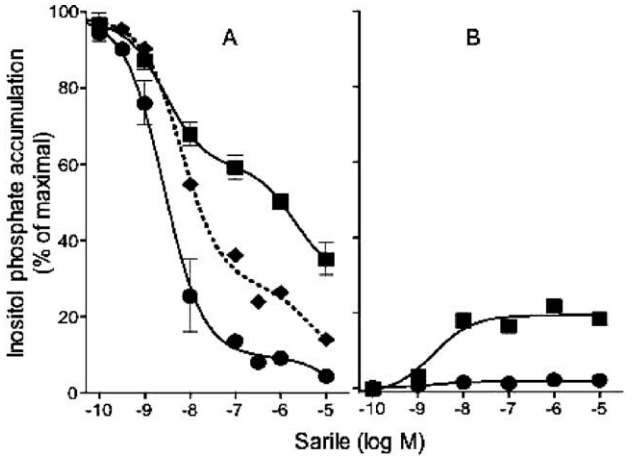

Fig. 5. (A) Concentration–inhibition curves of sarile on transient (■) and stable (●) cells. Cells were pre-incubated with increasing concentrations of sarile at 37 °C for 30 min and then stimulated by 10 μM angiotensin II for 15 min. (B) Concentration–response curves of sarile on transient (■) and stable (●) cells. Cells were incubated with increasing concentrations of sarile for 15 min. Data are expressed in percent of control (i.e., maximal stimulation by 10 μM angiotensin II). The dotted line represents the simulated curve of sarile for stable cells in the assumption that they contain the receptor reserve.

3.5. Binding studies with [^{125}I]sarile

In kinetic studies, the association and dissociation of 0.5 nM [^{125}I]sarile proceeded substantially faster for the transient cells than for the stable cells (Fig. 6). Since sarile and angiotensin II have been reported to induce angiotensin

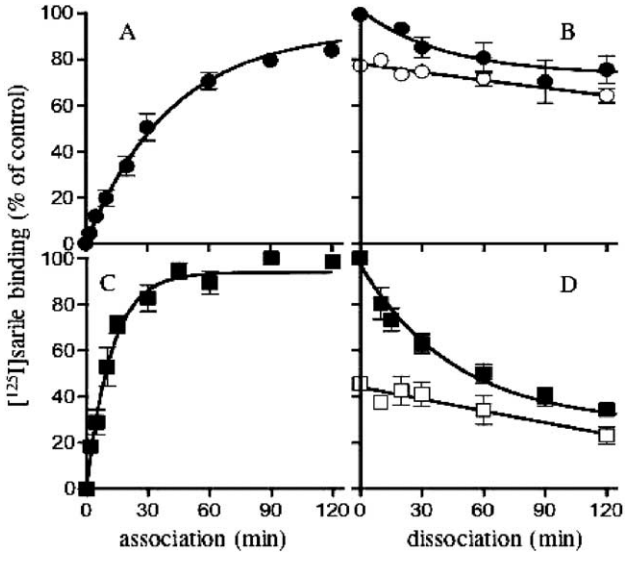

Fig. 6. Association (A, C) and dissociation (B, D) experiments of [^{125}I]sarile binding on stable (●) and transient cells (■). Cells were incubated with 0.5 nM [^{125}I]sarile in Hepes–DMEM containing 0.1% BSA at 37 °C for different period of time for association rate. Dissociation of [^{125}I]sarile from the cells was started by the addition of 1 μM unlabelled candesartan. After washing out the unbound ligand, both acid-resistant and acid-sensitive [^{125}I]sarile were collected. The acid-resistant bound [^{125}I]sarile associated with internalized receptors are expressed as a percentage of the total binding (acid-resistant plus acid-sensitive) for stable (○) and transient cells (□).

AT_1 receptor internalization in intact cells (Conchon et al., 1994; Hunyady et al., 2001), we investigated the possible impact of this process on the dissociation of [^{125}I]sarile. In the previous studies, cell surface-bound radioligand was swiftly removed by a mild acidic treatment while the acid-resistant binding was regarded to represent internalized radioligand. As shown in Fig. 6B and D, approximately 80% of the specific binding of [^{125}I]sarile was acid-resistant in the stable cells while this fraction was less than 50% in the transient cells. In both cell systems, the acid-resistant binding of [^{125}I]sarile dissociated extremely slowly while, comparatively, the acid-sensitive binding dissociated appreciably faster. Dissociation of the acid-sensitive binding from the transient cells was exponential with a rate constant of 0.035 ± 0.015 min^{-1}. Due to the low proportion of acid-sensitive binding, a similar analysis could not be performed for the stable cells.

Saturation binding experiments confirmed that the proportion of acid-resistant [^{125}I]sarile binding was more pronounced for the stable cells (Fig. 7A) than for the transient cells (Fig. 7C). This discrepancy was fairly constant, irrespectively of the [^{125}I]sarile concentration. Saturation binding experiments with the full agonist [^{3}H]angiotensin II also revealed a higher proportion of acid-resistant binding for the stable cells (Fig. 7B) than for the transient cells (Fig. 7D) (i.e., 83% and 63% of the total binding at 20 nM [^{3}H]angiotensin II, respectively).

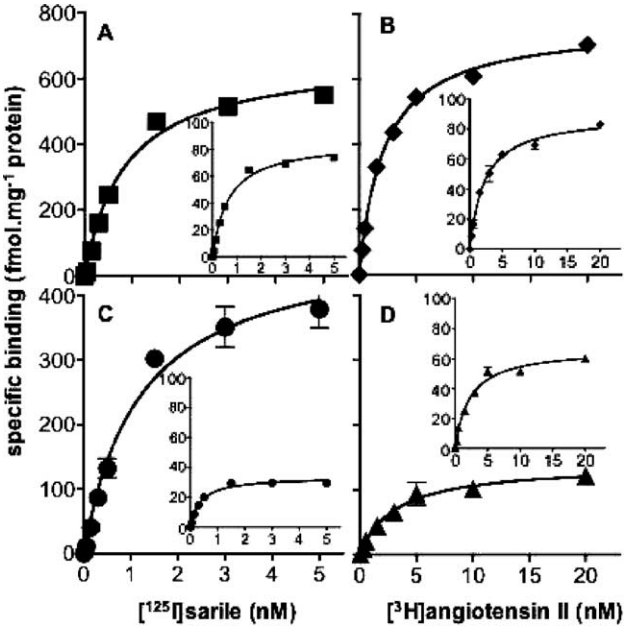

Fig. 7. Saturation and internalization of [^{3}H]angiotensin II and [^{125}I]sarile on the human angiotensin AT_1 receptor. Stable cells (A, B) and transient cells (C, D) were incubated with increasing concentrations of [^{125}I]sarile and [^{3}H]angiotensin II at 37 °C for 60 min. Specific binding was calculated by subtracting non-specific binding from total binding and was represented as fmole per milligram protein. Inserts show the corresponding internalized (acid-resistant) bound of the ligands, which express as a percentage of the total maximal binding (i.e., at highest concentration of the ligand).

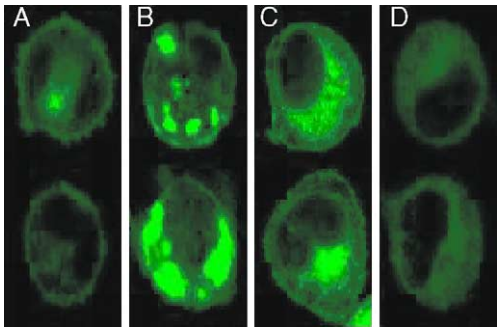

Fig. 8. Confocal imaging of the internalization of the human angiotensin AT_1 receptor. CHO-K1 cells transient expressed the EGFP–AT_{1A} receptor were stimulated with angiotensin II, sarile or candesartan as described in the Materials and methods section. Single cross-sections of focal planes were taken from the untreated cells (A) and from the cells treated with angiotensin II (B), sarile (C) and candesartan (D). The pictures are illustrated from 3 separated experiments.

3.6. Internalization of angiotensin EGFP-AT_{1A} receptor chimera

Angiotensin EGFP-AT_{1A} receptor chimera was transiently expressed in CHO-K1 cells to study the sub-cellular distribution of the receptors by confocal microscopy. Cells expressing a moderate level of receptor were chosen and cross sections with the largest nucleus diameter are shown in Fig. 8. In untreated cells, most of the EGFP fluorescence was found in the plasma membrane but some small spots were also detected in the nuclear envelope (Fig. 8A). After 15-min incubation with 10^{-7} M angiotensin II, the fluorescence became concentrated in large spots in the cytoplasm (Fig. 8B). A quite similar distribution of the fluorescence was observed after treatment of the cells with 10^{-7} M sarile (Fig. 8C) but the fluorescence pattern of the 10^{-8} M candesartan-treated cells was similar to the controls (Fig. 8D).

4. Discussion

Intact cell systems allow the investigation of ligand–receptor interactions by both radioligand binding and by the measurement of receptor-evoked responses. Very often, such studies involve the transient expression of the receptor-encoding gene in host cells which themselves do not express the receptor in question. In this respect, the present study reveals that some of the pharmacological and functional properties of transiently expressed AT_1 receptors CHO-K1 cells may differ from those of the stably expressed receptors in the same cell line.

According to the traditional pharmacological models, agonists should first produce a “stimulus” at the level of the receptor. The measured response should not only depend on the magnitude of this stimulus but also on the intermediate cascade of biochemical events within the cell. Amplification of the signal by this cascade may produce a non-linear

stimulus–response coupling, commonly denominated as "receptor reserve". In this respect, there appears to be a marked difference in the stimulus–response relationship between stably and transiently angiotensin AT_1 receptor expressing CHO-K1 cells. Analysis according to the method described by Umland et al. (2001) reveals a non-linear relationship in the transient cells with a half-maximal angiotensin II-induced inositol phosphate accumulation at 23% receptor occupancy. In contrast, this relationship is linear for the stable cells as evidenced from the 50% response at 49.9% receptor occupancy. For the stable cells, the same conclusion was already reached previously (Vauquelin et al., 2001a,c) based on kinetic experiments in where the angiotensin II-induced inositol phosphate accumulation was compared with the cell surface binding of [^{3}H]angiotensin II and [^{3}H]candesartan (Fierens et al., 1999b; Vanderheyden et al., 1999). Whereas the inositol phosphate accumulation closely followed the cell surface binding of [^{3}H]angiotensin II, there was an inverse relationship when comparing the receptor activity with the binding of [^{3}H]candesartan.

The difference in receptor reserve in both cell systems is sufficient to explain the higher potency of angiotensin II (Fig. 2A) as well as the lower degree of insurmountable inhibition by candesartan in the transient cells (Fig. 2B). Indeed, since [^{3}H]candesartan displays the same binding properties (i.e., association and dissociation rate constants, Fig. 3) for both cell systems, this latter observation cannot be attributed to the candesartan–receptor interaction. Instead, the behaviour of candesartan in both cell systems provides a nice illustration of the recent proposal by Lew et al. (2001) that the insurmountable behaviour of antagonists in functional studies should be diminished in systems with a hyperbolic stimulus–response relationship.

We have previously shown that pre-incubation of the stable cells with nonpeptide angiotensin AT_1 receptor antagonists like irbesartan, valsartan and EXP3174 only produced a partial decline in the maximal response by angiotensin II (Verheijen et al., 2000; Fierens et al., 2001). In the absence of receptor reserve, this decline is too small to be explained by the simple law of mass action (i.e., when the antagonists only form a single kind of complex with the AT_1 receptor) (Vauquelin et al., 2001c; Lew et al., 2001). So far, all experimental data with nonpeptide antagonists can be explained by a model in where (a) antagonist–receptor complexes may adopt fast reversible/surmountable and slow reversible/insurmountable binding states and (b) the equilibrium between these two states depends on the nature of the antagonist (Vauquelin et al., 2001a,b,c; Fierens et al., 2001; Verheijen et al., 2003).

A second major difference between stably and transiently expressed angiotensin AT_1 receptors is that sarile fails to produce noticeable receptor activation in the stable cells but that it clearly behaves as a partial agonist in the transient cells (with a maximal response corresponding to 19% of the one generated by angiotensin II). For the CHO cell system, this difference is too pronounced to be explained in terms of "receptor reserve" alone. Therefore, it is likely to reflect a difference in the propensity of stably and transiently expressed angiotensin AT_1 receptors to be stimulated by sarile. In other words, whereas the sarile-bound transiently expressed angiotensin AT_1 receptors may produce a measurable stimulus, the sarile-bound stably expressed receptors are unable to do so. As sarile also behaved as a partial agonist for recombinant HEK-293 and COS-1 cells transiently expressing angiotensin AT_1 receptors (Noda et al., 1995; Hines et al., 2001; Miserey-Lenkei et al., 2001), the present findings suggest that the stimulatory behaviour of sarile is governed by the mode of angiotensin AT_1 receptor transfection.

A third difference between both cell systems is the more pronounced internalization of [^{3}H]angiotensin II and [^{125}I]sarile in the stably expressing cells. In this respect, angiotensin II–AT_1 receptor complexes are known to swiftly dissociate in acidic pH and, in binding studies on intact cells, this property is commonly exploited to discriminate between the membrane-associated and internalized forms of the agonist. Exposure of the cells to a cold, mildly acidic buffer removes the radiolabel agonist from the cell surface. The residual, acid-resistant binding corresponds to internalized angiotensin II which is either still present in the endosomes together with the receptor or which has been directed to the lysosomes for its degradation (Hein et al., 1997; Holloway et al., 2002). Based on this approach, it appears that an appreciable proportion of bound [^{3}H]angiotensin II is internalized after 60-min incubation with both cell systems and that this proportion is higher for the stable cells than for the transient ones (Fig. 7B,D). In agreement with earlier studies (Crozat et al., 1986; Conchon et al., 1994; Thomas et al., 1996) a large amount of bound [^{125}I]sarile was also found to be acid-resistant in stably and transiently angiotensin AT_1 receptor expressing CHO-K1 cells (Fig. 6). Here again, the proportion of acid-resistant binding was more pronounced for the stable cells than for the transient ones. To ascertain that sarile may effectively internalize angiotensin AT_1 receptors in CHO-K1 cells, we exposed angiotensin EGFP-AT_{1A} receptor chimera expressing cells to angiotensin II, sarile and candesartan. Whereas the fluorescence became confined in large spots in the cytoplasm for both angiotensin II and sarile, no major modification of the fluorescence pattern was observed in the case of candesartan (Fig. 8). In agreement, other recent confocal microscopic studies also established that sarile as well as related Phe^8 analogs of angiotensin II and other pseudopeptides like CGP42112A provoke the internalization of angiotensin AT_1 receptor–EGFP conjugates (Miserey-Lenkei et al., 2001; Holloway et al., 2002). As this process occurs independently of their agonist or antagonist properties, it has been proposed that occupancy of the peptide binding site of the angiotensin AT_1 receptor may trigger its internalization and that the involved receptor conformation differs from the one required for receptor

activation and signal transduction (Miserey-Lenkei et al., 2001; Holloway et al., 2002). In contrast, the present as well as previous studies with the nonpeptide antagonists candesartan, losartan and EXP3174 indicate that this class of ligands is unable to trigger angiotensin AT_1 receptor internalization (Hein et al., 1997; Miserey-Lenkei et al., 2001; Hunyady et al., 2001; Fierens et al., 2001; Holloway et al., 2002).

The molecular mechanisms that are responsible for the abovementioned disparities between stably and transiently expressed angiotensin AT_1 receptors are presently unknown but it is reasonable to assume that it could involve differences in their maturation and/or their cellular surrounding. Interestingly, clear differences in maturation (involving processes like protein folding, post-translational modification and transport through distinct cellular compartments) have already been observed when comparing stably and transiently expressed vasopressin V2 receptors and gastric H,K-ATPase–heterodimer fusion proteins (Sadeghi et al., 1997, 1998; Lambrecht et al., 1998). Whereas the vasopressin V2 receptors synthesized in transiently transfected HEK293 cells were mainly immature, stably transfected cells produced almost exclusively mature receptor proteins. Furthermore, the maturation of this receptor was found to be unrelated to glycosylation suggesting that it was the consequence of protein refolding (Sadeghi et al., 1997, 1998). As second example, the stable transfection of HEK 293 cells with the gene coding for a gastric H,K-ATPase–heterodimer fusion protein produced a protein with a complex glycosylation pattern and with a correct plasma membrane localization. On the other hand, transient transfection with the same gene gave rise to a protein with only core glycosylation and minor ATPase activity. Furthermore, this protein remained mainly located in the endoplasmic reticulum (Lambrecht et al., 1998).

Differences in angiotensin AT_1 receptor maturation may affect their conformation and their degree of phosphorylation and, as mutation studies suggest, this might have profound consequences for their ligand binding and functional properties. Of special interest in this respect is the increased responsiveness of sarile when comparing the constitutively active Asn^{111} mutated angiotensin AT_1 receptor with its wild-type counterpart in CHO-K1 and COS-1 cells (Noda et al., 1996; Le et al., 2002). These receptor mutants are no longer endowed with a constraining interaction between Asn^{111} and TM VII and they are therefore generally regarded to mimic a pre-activated state of the angiotensin AT_1 receptor (Noda et al., 1996; Balmforth et al., 1997; Groblewski et al., 1997; Le et al., 2002). Whereas constitutively active receptor mutants represent well-known examples of facilitated G-protein-coupled receptor activation, other angiotensin AT_1 receptor mutants have now also been shown to display facilitated activation by the peptide ligand CGP42112A even without noticeable increase of their basal activity (Parnot et al., 2000). Initial angiotensin AT_1 receptor mutation studies pointed at a casual link between angiotensin II-mediated receptor phosphorylation and its internalization. Indeed, the selective replacement of serine and threonine residues in the central part of the angiotensin AT_1 receptor carboxyl terminus as well as the progressive truncation of this terminus resulted in a concerted decrease in receptor internalization and phosphorylation (Smith et al., 1998; Thomas et al., 1998). However, subsequent studies with wild-type and Asn^{111} mutated receptors showed a more complicated situation in the case of substituted angiotensin II analogues. As these compounds sometimes display marked differences in their capacity to promote receptor signalling, phosphorylation and internalization, it is now proposed that angiotensin AT_1 receptors are capable to adopt different ligand-specific states/conformations and that these may show preference with regard to triggering distinct receptor activities (Thomas et al., 2000; Holloway et al., 2002). This multi-active receptor conformation hypothesis fits nicely with the two-state model for nonpeptide antagonist–receptor interaction (Fierens et al., 1999b; Vauquelin et al., 2001a) and with the current thinking that receptors exist in a very large number of conformational states, which may be regarded to constitute minima of an "energy landscape" (Kenakin, 1996). This may also explain the more pronounced internalization of [^{125}I]sarile in the stable cells despite the fact that this ligand only produces perceptible receptor stimulation in the transient cells.

Finally, it has also been proposed that the capacity of angiotensin II and related peptides to promote angiotensin AT_1 receptor signalling and internalization may be affected by its cellular environment. In this respect, intrinsic membrane proteins like β-adrenergic receptors were originally regarded to float in a homogeneous sea of lipids (De Lean et al., 1980). However, the existence of cholesterol-enriched membrane microdomains like lipid rafts and caveolae is now well recognised and evidence is now also accumulating for their role in cell signalling either by bringing signalling partners (such as G-protein-coupled receptors and G proteins) together or by inducing their spatial segregation (Pike, 2003). In accordance with this rejuvenated model of the plasma membrane, several recent studies have established that angiotensin AT_1 receptors are unequally distributed across this membrane but there is still some controversy regarding the implication of lipid rafts versus caveolae in this phenomenon (Ishizaka et al., 1998; Leclerc et al., 2002; Wyse et al., 2003). In the light of these findings, it could be of interest to compare the membrane distribution of permanently and transiently expressed angiotensin AT_1 receptors. Indeed, differences in the membrane lipid environment and in the proximity of G proteins and other potential regulatory molecules may all affect the ligand binding and functional properties of such receptors (Pike, 2003; Verheijen et al., 2004). In this respect, differences in the amount of "prearranged" angiotensin AT_1 receptor–G_q protein signalling complexes could explain the existence of a "receptor reserve" in the transient cells despite

the fact that the actual angiotensin AT_1 receptor concentration is higher in the stable cells. Moreover, angiotensin AT_1 receptors have also been shown to interact specifically with ATRAP, a 3 membrane-spanning domain protein (Cui et al., 2000; Lopez-Ilasaca et al., 2003). Since ATRAP is able to decrease the angiotensin AT_1 receptor-mediated inositol phosphate generation and to enhance receptor internalization, it is also plausible that the angiotensin AT_1 receptor–ATRAP interaction is favoured in the stable cells.

In conclusion, we observed that some of the pharmacological and functional properties of transiently expressed angiotensin AT_1 receptors CHO-K1 cells differ from those of the stably expressed receptors in the same cell line. They include the ability of sarile to produce measurable angiotensin AT_1 receptors stimulation and the existence of a non-linear stimulus–response coupling (i.e., "receptor reserve") in the transient cells only. Both properties are absent in the stable cells and in rabbit aortic strips (the prototype tissue for the investigation of angiotensin AT_1 receptors) (Liu et al., 1992; Zhang et al., 1994). Hence, the stable angiotensin AT_1 receptors-expressing CHO-K1 cells are likely to provide the most relevant information from the physiological point of view. The molecular mechanisms underlying the differences in angiotensin AT_1 receptor behaviour in transient and stable cells are presently unknown but it is reasonable to assume that it could involve differences in receptor maturation and/or in their surrounding. It is therefore pertinent to take account of the mode of receptor (and other protein) expression when comparing its characteristics with data provided in other studies.

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