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Tight binding of the angiotensin AT_1 receptor antagonist [^{3}H]candesartan is independent of receptor internalization

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Abstract

Angiotensin II induces angiotensin AT_1 receptor internalization via Clathrin coated pits formation. We investigated whether insurmountable inhibition by the non-peptide antagonist 2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl) biphenyl-4-yl) methyl]-1*H*-benzimidazoline-7-carboxylic acid (candesartan) was related to receptor internalization. Mild acid treatment can discriminate between internalized and cell surface bound [^{3}H]angiotensin II. In contrast, it provides no information about the subcellular localization of bound [^{3}H]candesartan since this binding is acid resistant. The internalization of [^{3}H]angiotensin II is rapidly inhibited in the presence of 0.4 M sucrose. Yet, no such rapid effect was noticed for [^{3}H]candesartan. [^{3}H]candesartan displays insurmountable/long lasting binding to the vast majority of both wild type and L^{314} truncated rat angiotensin AT_{1A} receptors with impaired receptor internalization. In agreement with previously published AT_1 angiotensin receptor visualization experiments, the present data suggest that non-peptide antagonist-angiotensin AT_1 receptor complexes remain at the cell surface. Insurmountable antagonism of candesartan is therefore independent from receptor internalization via clathrin-coated pits.

Keywords: Candesartan; Angiotensin AT_1 receptor; CHO cells; Insurmountable antagonist; Internalization; Sucrose

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 [1]. These actions of angiotensin II are mediated by receptors of the angiotensin AT_1 subtype. A second receptor subtype, the angiotensin AT_2 receptor, which is highly expressed in fetal tissues, has been cloned recently [2,3] but its functions are still ill defined. The interaction between the angiotensin AT_1 receptor and its antagonists has often been studied by in vitro contraction experiments on rabbit aorta and rat portal vein. These experiments comprise a preincubation step, in which the tissue is pre-equilibrated with the antagonist, and an incubation step with consecutive additions of increasing concentrations of angiotensin II to generate a dose-response curve. Two categories of antagonists are usually distinguished. Whereas surmountable antagonists such as 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)-biphenyl-4-yl)-methyl]-imidazole (losartan) produce parallel rightward shifts of the dose-response curve, insurmountable antagonists produce a limited (e.g. 2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl)-biphenyl-4-yl)-methyl]-imidazole-5-carboxylic acid (EXP3174), 2-n-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)-biphenyl-4-yl)-methyl]2-imidazolin-5-one (irbesartan)) or almost full (e.g. candesartan) depression of the maximal response [4–6].

Recent experiments on Chinese hamster ovary cells stably transfected with the human angiotensin AT_1 receptor

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Abbreviations: candesartan, 2-ethoxy-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benzimidazoline-7-carboxylic acid; CHO-K1, Chinese hamster Ovary cells; CHO-hAT_1 cells, CHO-K1 cells expressing human AT_1 receptors; CHO-rAT_{1A}-WT, CHO-K1 cells expressing wild type rat AT_{1A} receptors; CHO-TL_{314}-rAT_{1A}, CHO-K1 cells expressing rat AT_{1A} receptors with a truncated cytoplasmic tail at Leucine 314; HEPES, N-[2-hydroxyethyl]piperazine-N′-[ethanesulfonic acid]; IP, inositol mono-, bis- and trisphosphates.

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(CHO-hAT_1 cells), revealed that angiotensin AT_1 receptor antagonists are competitive with angiotensin II. It was found that antagonist-angiotensin AT_1 receptor complexes can adopt a fast dissociating/surmountable state and a slow dissociating/insurmountable state, and that the equilibrium between both states is dependent on the nature of the antagonist [7,8]. The distinction between both states could reside at the level of the receptor conformation, its association with other proteins or even its sub-cellular localization. In line with this, Liu *et al*. [4] invoked the ability of angiotensin AT_1 receptor antagonists to exert a direct or indirect control on the internalization of their receptors. This could explain the fact that most of these insurmountable antagonists only confer a limited depression of the maximal response to angiotensin II in contraction studies. This proposal also resides from the fact that angiotensin AT_1 receptors, when stimulated by agonists, are particularly prone to internalize into the cell as part of their recycling process. For angiotensin II, it has been established that it binds first to angiotensin AT_1 receptors at the cell surface and that the agonist-receptor complexes are rapidly internalized via coated pits into endosomes. The agonist is then targeted to the lysosomal pathway while the receptors are continuously recycled to the plasma membrane [9,10].

The binding of angiotensin II to the angiotensin AT_1 receptor is known to be very sensitive to acidic pH. This property is commonly exploited in binding studies on intact cells to discriminate between the membrane-associated and internalized forms of the agonist [11–13]. Brief exposure of the cells to an acidic buffer removes the radiolabeled agonist from the cell surface and the internalized agonist accounts for the residual, acid-resistant binding [10]. Based on the finding that the binding of radiolabeled angiotensin AT_1 receptor antagonists to intact cell systems is also more or less acid resistant, Crozat *et al*. [11] and Conchon *et al*. [14] evoked the possibility that antagonist-angiotensin AT_1-receptor complexes internalize and may account for this acid resistant binding.

To investigate the potential link between tight antagonist binding (insurmountable antagonism) and receptor internalization, candesartan constitutes the antagonist of choice because of its ability to undergo tight, long lasting binding to a large proportion of the angiotensin AT_1 receptors. In the present report, two approaches were applied. 1) The effect of preventing 7 transmembrane receptor endocytosis via coated pit formation, by exposing cells to a hypertonic sucrose concentration [15], was measured on [^{3}H]candesartan binding. 2) The insurmountable antagonism of candesartan was studied on CHO-TL_{314}-rAT_{1A} cells. These CHO-K1 cells expressing rat AT_{1A} receptors with a truncated cytoplasmic tail at leucine 314, have previously been shown by Thomas *et al*. [16] to exhibit most characteristics of the wild type receptor, except for a significantly reduced capacity for agonist-induced internalization.

2. Materials and methods

2.1. Drugs and cells used

Candesartan [17,18] and [^{3}H]candesartan (17 Ci/mmol) were obtained from AstraZeneca. Unlabeled angiotensin II was obtained from Sigma. [^{3}H]angiotensin II (20 Ci/mmol) was obtained from New England Nuclear and *myo*-[^{3}H]-inositol (20 Ci/mmol) was from Pharmacia/Amersham/Biotech. All other chemicals were of the highest grade commercially available. CHO-hAT_1 cells were obtained as described by Vanderheyden *et al*. [19]. CHO-rAT_{1A}-WT and CHO-rAT_{1A}-TL_{314} cells were generated as described previously [16,20]. The cells were cultivated, as described previously [19].

2.2. Radioligand binding on intact CHO cells

The cell surface and acid resistant binding of [^{3}H]angiotensin II and [^{3}H]candesartan to the different cell lines has been measured on adherent cells in 24-well plates as described previously [7,19]. The radioligands were incubated at 37° for 30 min (saturation and dissociation experiments) in HEPES-buffer (HEPES 20 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 2.1 mM, NaCl 137 mM, KCl 2.7 mM, pH 7.4). In the kinetic experiments [^{3}H]candesartan dissociation started upon addition of 1 μM unlabeled candesartan and continued for the indicated periods of time. For the sucrose experiments HEPES-buffer was used containing 0.4 M sucrose or the indicated final concentrations for the given periods of time. Treatment with ice-cold Glycine buffer (50 mM Glycine, 125 mM NaCl at pH 3.0, 2 $\times$ 5 min) (mild acid) and subsequent NaOH lysis of the cells was performed as described previously [19]. The radioactivity was counted in a liquid scintillation counter. The protein content was approximately 0.13 $\pm$ 0.03 mg/well.

2.3. Radioligand binding on CHO-hAT_1 cell membranes

Cultured cells were harvested by trypsinization (0.05% trypsine, 0.02% EDTA in Phosphate buffered Saline). The harvested cells were washed three times with HEPES-buffer (4°, 5 min at 500 *g*) and homogenized by Polytron and Potter homogenizer. The resulting homogenate was centrifuged (4°, 30 min at 27.000 *g*) and the supernatant was removed. The pellet was resuspended in ice-cold HEPES-buffer. The radioligand binding assays were performed with 400 μL membrane suspension ($\approx$0.53 mg protein/mL). For the sucrose experiments HEPES-buffer was used containing 0.4 M sucrose. Control experiments revealed that the harvesting procedure (trypsinization or scraping) did not influence the [^{3}H]candesartan binding.

Inhibition experiments were initiated by incubating the membranes in the presence or not (control) of 0.4 M sucrose in HEPES-buffer at 37° for 30 min. Subsequent incubations for 30 min started by adding 50 μL of HEPES-buffer either

alone (total binding) or containing unlabelled competitor (competition binding) and 50 μL of HEPES-buffer containing 1.5 nM [^{3}H]candesartan. Kinetic experiments were started by adding 50 μL of HEPES-buffer containing 1.5 nM [^{3}H]candesartan (final concentration) to the membranes. Incubations continued for 30 min (dissociation experiments) or the indicated periods of time (association experiments). Dissociation of [^{3}H]candesartan was started upon addition of 1 μM unlabeled candesartan and continued for the indicated periods of time. To determine the sensitivity to Glycine buffer, the membranes were centrifuged and resuspended in 500 μL of ligand-free, ice-cold HEPES or Glycine buffer and further incubated on ice for 0, 5, 10 or 15 min. Non-specific binding was determined by adding 1 μM (final) unlabeled candesartan just before [^{3}H]candesartan. The same values for non-specific binding were obtained using 1–10 μM unlabelled angiotensin II (data not shown). The incubations were stopped by adding 3.0 mL ice-cold HEPES-buffer. The membranes were subsequently filtered and washed on Whatmann GF/B filters. The filters were placed in vials, 3.0 mL of scintillation liquid (Optisafe of Wallac, Turku, Finland) was added and the samples were counted for 3 min in a liquid scintillation counter.

2.4. [^{3}H]thymidine uptake

The cells were plated in 24-well plates and cultured until confluent (see above). The cells were washed and incubated overnight in growth medium without fetal bovine serum (5% CO_2 at 37°). Before the experiment cells were washed with Dulbecco's Modified Essential Medium buffer. The time-wise effect of hypertonic sucrose (0.4 M) on [^{3}H]thymidine uptake was measured by incubating the cells with sucrose for the indicated periods of time. 15 min before the end of the incubation 100 nM [^{3}H]thymidine was added. As negative control 10% ethanol was added just before the [^{3}H]thymidine addition. The radioactivity measured in these cells was regarded as 'non-specific' [^{3}H]thymidine uptake. After the incubation cells were placed on ice, washed three times with ice cold HEPES buffer and three times with ice-cold 10% trichloric acetic acid, and lysed with 0.4 M NaOH. Radioactivity was measured like on intact cells.

2.5. IP accumulation

The angiotensin II induced IP accumulation in the presence of 10 mM LiCl was determined on adherent cells in 24-well plated as described by Vanderheyden *et al.* [19] and represents the formation of the inositol mono-, bi-, and triphosphates.

2.6. Data treatment

IC_{50} values from competition binding experiments, association and dissociation rate constants from kinetic experiments and the K_d and the B_{max} values from saturation binding curves were calculated by non-regression analysis using GraphPad Prism.

Table 1
Effect of sucrose on [^{3}H]angiotensin II and [^{3}H]candesartan binding; acid-resistance vs acid-sensitivity

		Total (%)	Acid sensitive (%)	Acid resistant (%)
[^{3}H]angiotensin II	Control	100.0[a]	16.1 ± 1.8	83.8 ± 1.8
	0.4 M sucrose	51.1 ± 5.9	39.7 ± 5.5	11.4 ± 3.7
[^{3}H]candesartan	Control	100.0[b]	4.3 ± 0.9	97.6 ± 0.8
	0.4 M sucrose	75.4 ± 3.1	5.4 ± 1.1	73.3 ± 2.9

Intact CHO-hAT_1 cells were preincubated during 15 min with sucrose or buffer (control). Subsequently 1.0 nM [^{3}H]angiotensin II or 1.5 nM [^{3}H]candesartan was added for 30 min. The represented values are the mean ± SEM of at least 5 independent experiments, three one-concentration determinations each.

[a]1.0 nM [^{3}H]angiotensin II control binding equaled 325 ± 82 fmol/mg protein.

[b]1.5 nM [^{3}H]candesartan control binding equaled 712 ± 163 fmol/mg protein.

3. Results

3.1. [^{3}H]angiotensin II and [^{3}H]candesartan binding to intact CHO-hAT_1 cells

When CHO-hAT_1 cells were incubated for 30 min with 1.0 nM [^{3}H]angiotensin II at 37°, 16% of its specific binding could be extracted upon brief exposure with ice-cold Glycine buffer (pH 3.0) (Table 1). The remaining acid resistant binding could be extracted by treating the cells with 0.4 M NaOH. For [^{3}H]candesartan almost all of the specific cell binding was acid resistant (97%) (Table 1). Internalization of AT_1 receptors via clathrin-coated pits was effectively prevented by exposing the cells to a hypertonic concentration of sucrose [15]. When given to the CHO-hAT_1 cells, 0.4 M sucrose produced an immediate increase of 24% of the specific acid-sensitive [^{3}H]angiotensin II binding and a pronounced decline in acid-resistant binding (Table 1). In contrast, the [^{3}H]candesartan binding declined slightly but remained acid-resistant in the presence of 0.4 M sucrose.

The sucrose-mediated decline of the specific [^{3}H]angiotensin II and [^{3}H]candesartan binding was time-dependent. In the experiment shown in Fig. 1, the CHO-hAT_1 cells were exposed for increasing periods of time with 0.4 M sucrose and, in the continued presence of sucrose, further incubated with the radioligands during the last 30 min. Under these conditions, acid-sensitive [^{3}H]angiotensin II binding decreased time-wise to undetectable levels after 120 min sucrose exposure (Fig. 1a). The acid-resistant binding was almost completely decreased at the shortest incubation time with sucrose. In contrast, specific [^{3}H]candesartan

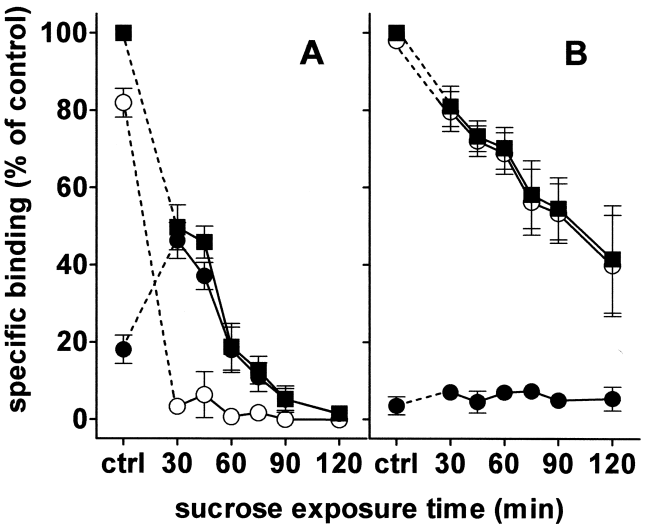

Fig. 1. Time–dependency of the sucrose effect (0.4 M) on CHO-hAT$_1$ cells measured after the different sucrose pre-incubation times i.e. the acid-sensitive binding (●), the remaining acid-resistant binding (○) and the total specific cell binding (■) of 1.0 nM [^{3}H]angiotensin II binding (A) and 1.5 nM [^{3}H]candesartan binding (panel B). Data are mean values ± SEM of three independent experiments, two determinations each and represented as % of the initial total radioligand binding at time 0 min (i.e. control) in the absence of sucrose.

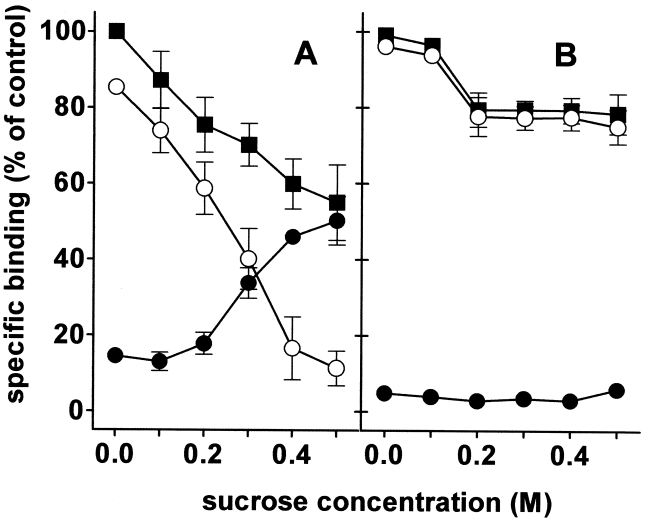

Fig. 2. Concentration dependency of the sucrose effect on the total specific cell binding (■), the acid-sensitive binding (●) and the remaining acid-resistant binding (○) of 1.0 nM [^{3}H]angiotensin II binding (panel A) and 1.5 nM [^{3}H]candesartan binding (panel B). CHO-hAT$_1$ cells were washed and preincubated for 15 min. Subsequently the radioligand was added and the CHO-hAT$_1$ cells were further incubated for 30 min (i.e. total sucrose-incubation time is 45 min). Data are mean values ± SEM of 3–5 independent experiments, two determinations each and represented as % of the total radioligand binding in the absence of sucrose (0.0 M sucrose i.e. control).

binding remained acid-resistant and was only decreased by 58% after 120 min exposure of the cells to sucrose (Fig. 1b).

The effect of sucrose was also concentration-dependent. A 45-min incubation with sucrose produced a concentration-wise reduction of the specific [^{3}H]angiotensin II binding. This resulted from a large decrease in acid-resistant binding and a less pronounced increase in the acid-sensitive binding (Fig. 2a). These effects were nearly maximal at 0.4 M sucrose. Sucrose also produced a concentration-wise decline of the specific [^{3}H]candesartan binding up to 20%, but all of it remained acid-resistant (Fig. 2b).

Sucrose did not influence the association kinetics of 1.5 nM [^{3}H]candesartan binding to CHO-hAT$_1$ cells and the equilibrium was reached after 20 min. At a constant exposure period of the cells (45 min) to 0.4 M sucrose, there was a 20% decrease of B_{eq} of the [^{3}H]candesartan binding but the apparent first order rate constant remained unchanged (i.e. $k_{obs} = 0.20 \pm 0.04$ min^{-1} in the presence of sucrose versus 0.23 ± 0.03 min^{-1} in medium only). The effect of sucrose on [^{3}H]candesartan dissociation kinetics could not be measured because of the long half-life of the radioligand-AT$_1$ receptor complex [7].

3.2. [^{3}H]thymidine uptake in CHO-hAT$_1$ cells

The reduction of specific [^{3}H]angiotensin II and [^{3}H]candesartan binding by sucrose was not due to detachment of CHO-hAT$_1$ cells from the microwell plates as the protein content per well was not affected by sucrose treatment (0.4 M for 90 min; data not shown). The cell viability and proliferation was assessed by measuring [^{3}H]thymidine uptake. For this purpose, cells were kept overnight in culture medium without serum, followed by an incubation with 100 nM [^{3}H]thymidine. In the absence of sucrose, there was a linear time-dependent increase of [^{3}H]thymidine uptake in CHO-hAT$_1$ cells. As shown in Fig. 3, sucrose caused a time-dependent inhibition of the capability to incorporate [^{3}H]thymidine. The uptake was almost completely inhibited after treatment of the cells with 0.4 M sucrose for 30 min.

3.3. [^{3}H]candesartan binding to CHO-hAT$_1$ cell membranes

In contrast to intact CHO-hAT$_1$ cells, the specific binding of [^{3}H]candesartan to the cell membranes was not affected by 0.4 M sucrose. The association and the dissociation rates and the calculated K_d of [^{3}H]candesartan (Fig. 4) as well as the K_i values of unlabeled angiotensin II and candesartan calculated from competition binding experiments (Table 2) revealed no difference in the absence or presence of 0.4 M sucrose. The sensitivity of [^{3}H]candesartan binding to mild acid treatment was also checked. Membranes were incubated for 30 min with 1.5 nM [^{3}H]candesartan, centrifuged, resuspended in fresh ice-cold HEPES-buffer (pH 7.4) or Glycine buffer (pH 3.0) and further incubated on ice for 0, 5, 10, and 15 min. No substantial dissociation of [^{3}H]candesartan was observed in both buffers (i.e. HEPES- and Glycine buffer) (Fig. 5).

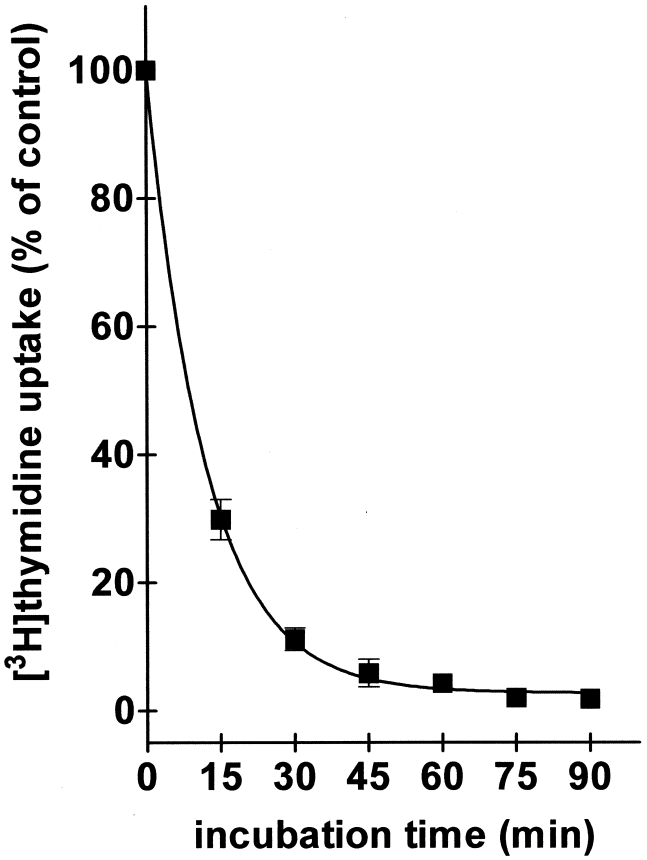

Fig. 3. The time–dependency of the sucrose effect (0.4 M) on [^{3}H]thymidine uptake (100 nM, 15 min) in CHO-hAT1 cells (■). The data are mean values ± SEM of three independent experiments, three determinations each and represented as % of the control [^{3}H]thymidine uptake in the absence of sucrose. 100% [^{3}H]thymidine uptake corresponded to 13400 ± 2040 dpm/well, whereas 0% (in the presence of 10% ethanol) corresponded to 322 ± 50 dpm/well.

3.4. Binding to CHO-K1 cells expressing the wild-type or L^{314}-truncated rat AT_{1A} receptor

[^{3}H]angiotensin II and [^{3}H]candesartan binding to intact CHO-rAT_{1A}-WT and CHO-TL_{314}-rAT_{1A} cells are compared in Fig. 6. The truncated receptors have previously been shown to exhibit most characteristics of the wild type receptor, except for a significantly reduced capacity for agonist-induced internalization [16]. In agreement, the majority of the bound [^{3}H]angiotensin II could be extracted from CHO-TL_{314}-rAT_{1A} cells with a mild acid buffer (Fig. 6b). This was opposite to CHO-rAT_{1A}-WT (Fig. 6a) and CHO-hAT_1 cells (Table 1) for which the majority of bound [^{3}H]angiotensin II was acid-resistant. Similar to CHO-hAT_1 cells, the binding of [^{3}H]candesartan to intact CHO-rAT_{1A}-WT and CHO-rAT_{1A}-TL_{314} cells was exclusively found in the acid-resistant fraction (Fig. 6c,d). B_{max} values derived from saturation binding curves of [^{3}H]angiotensin II and [^{3}H]candesartan are 167 ± 7 and 156 ± 5 fmol/mg protein on intact CHO-rAT_{1A}-WT and 103 ± 8 and 98 ± 4 fmol/mg protein CHO-rAT_{1A}-TL_{314} cells (panel 6b and 6d)

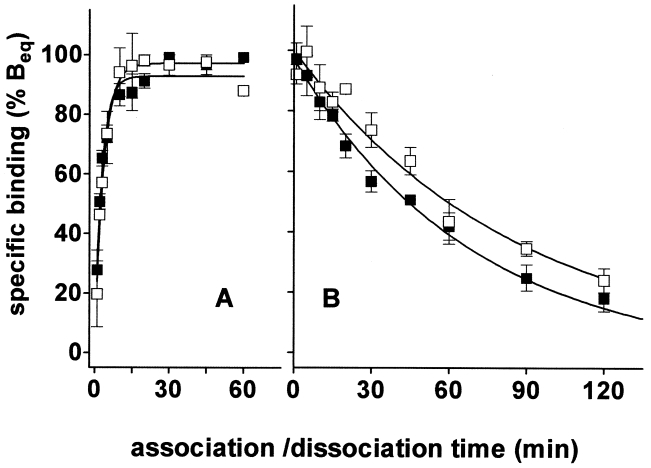

Fig. 4. Association (A) and dissociation (B) of [^{3}H]candesartan (1.5 nM) on CHO-hAT_1 cell membranes in the absence (■) or presence of 0.4 M sucrose (□). The calculated dissociation (k_{-1}) and association (k_{+1}) rate constants were respectively 0.018 ± 0.004 min^{-1} and 0.32 ± 0.06 $min^{-1}nM^{-1}$ in the absence and 0.012 ± 0.001 min^{-1} and 0.26 ± 0.08 $min^{-1}nM^{-1}$ in the presence of sucrose. These parameters gave a Kd of 0.20 ± 0.04 nM and 0.17 ± 0.05 nM in the absence or presence of 0.4 M sucrose. The data are represented as % of the maximal specific binding in the absence of sucrose i.e. control (690 ± 250 fmol/mg protein). Dissociation was initiated by addition of 1 μM of unlabelled candesartan after a 30 min [^{3}H]candesartan incubation.

respectively. Kinetic experiments, in which the dissociation of bound [^{3}H]candesartan was initiated by isotopic dilution (by adding 1.0 μM unlabelled candesartan) showed no substantial difference in the dissociation rate constants of the [^{3}H]candesartan between both cell lines. Dissociation was exponential in both cases, with a k_{-1} of 0.0088 ± 0.0019 min^{-1} for CHO-rAT_{1A}-WT cells and 0.0053 ± 0.0022 min^{-1} for CHO-rAT_{1A}-TL_{314} cells ($N = 3$).

3.5. IP accumulation in CHO-rAT_{1A}-TL_{314} and CHO-rAT_{1A}-WT cells

Angiotensin II induced a dose-dependent increase of the IP production with similar potencies in CHO-rAT_{1A}-TL_{314}

Table 2
K_i values (nM) of candesartan and angiotensin II on CHO-hAT_1 cell membranes

K_i values (nM)	control	0.4 M sucrose
Candesartan	0.56 ± 0.33	0.30 ± 0.12
Angiotensin II	26.9 ± 16.7	9.4 ± 2.8

K_i values were calculated according to the Cheng and Prussoff equation [30] using the K_ds of [^{3}H]candesartan given in Fig. 4 and IC_{50}s derived from [^{3}H]candesartan inhibition curves on CHO-hAT_1 cell membranes were pre-incubated for 30 min with or without 0.4 M sucrose and subsequently incubated for 30 min with 1.5 nM [^{3}H]candesartan and increasing concentrations of unlabelled candesartan or angiotensin II. The represented calculated parameters were the mean ± SE of at least 3 independent experiments, three determinations each.

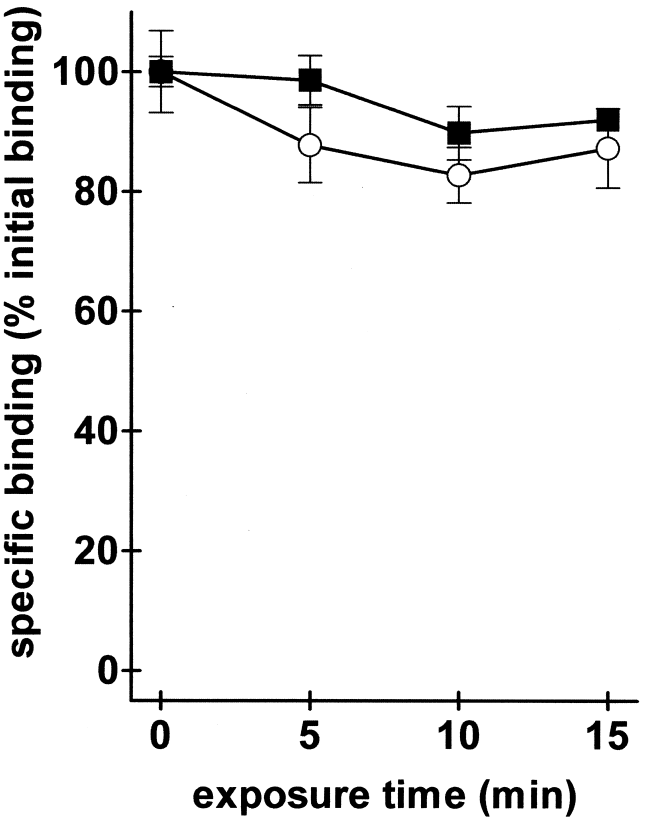

Fig. 5. Insensitivity of the [^{3}H]candesartan binding to mild acid. Isolated CHO-hAT_1 cell membranes were incubated for 30 min at 37° with 1.5 nM [^{3}H]candesartan in HEPES-buffer. Subsequently the membranes were centrifuged, resuspended in ice-cold HEPES buffer (■) or Glycine buffer (○) and further exposed for the indicated time on ice. The [^{3}H]candesartan control binding normalized to 100% equaled 1028 ± 193 fmol/mg protein. Experimental values represent the mean ± SEM of 3 independent experiments, 3 determinations each.

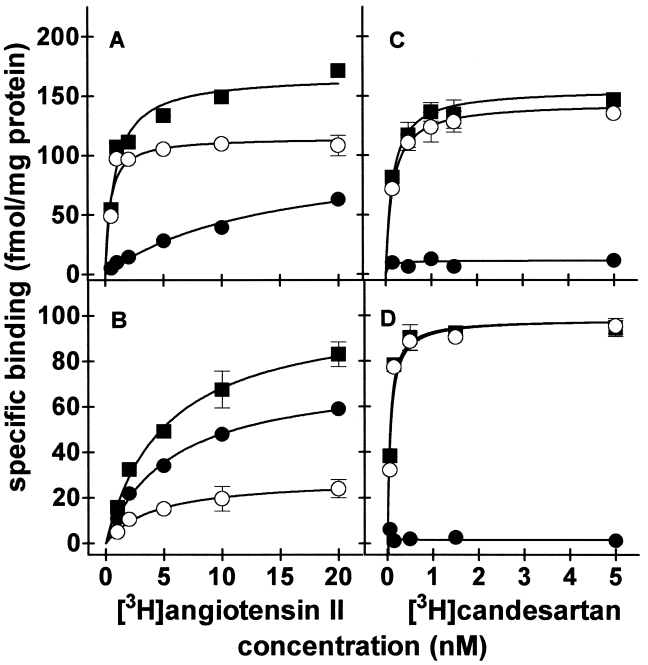

Fig. 6. [^{3}H]angiotensin II and [^{3}H]candesartan saturation binding data to intact CHO-rAT_{1A}-WT (A and C) and CHO-rAT_{1A}-TL_{314} cells (B and D). [^{3}H]angiotensin II and [^{3}H]candesartan were incubated for 30 min at 37°. The acid-sensitive binding (●), the remaining acid-resistant binding (○) and the specific cell binding (■) were measured and calculated. Data are mean values ± SEM of a typical example, in triple determination. B_{max} values of the [^{3}H]angiotensin II and [^{3}H]candesartan binding are 167 ± 7 and 156 ± 5 fmol/mg protein on intact CHO-rAT_{1A}-WT and 103 ± 8 and 98 ± 4 fmol/mg protein CHO-rAT_{1A}-TL_{314} cells (B and D), respectively.

and in CHO-rAT_{1A}-WT cells (EC_{50} = 10.0 ± 4.0 nM and 4.0 ± 2.0 nM respectively) (Fig. 7). A 30-min preincubation of both cell lines with 5.0 nM candesartan caused a similar, dramatic reduction of the maximal responses by angiotensin II. This insurmountable inhibition was also apparent when CHO-rAT_{1A}-TL_{314} and CHO-rAT_{1A}-WT cells were preincubated with increasing candesartan concentrations. As depicted in Fig. 7b the obtained concentration-inhibition curves were biphasic. The most potent component of these curves corresponded to insurmountable inhibition [8] and comprised 92% and 88% of the response in CHO-rAT_{1A}-TL_{314} and CHO-rAT_{1A}-WT cells, respectively.

4. Discussion

Morphological studies have shown that brief exposure of cells to an acidic buffer is sufficient to remove angiotensin II from its angiotensin AT_1 receptors that are present on 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 [10]. For human angiotensin AT_1 receptors transfected in CHO cells (CHO-hAT_1), it was shown that most of the bound [^{3}H]angiotensin II was already acid-resistant after 5-min incubation at 37° and that this form accounted for the steady increase of [^{3}H]angiotensin II binding with the incubation time [19]. This agrees with the very rapid internalization of angiotensin II in other cell types such as human embryonic kidney 293 cells stably expressing rat AT_{1A} receptors and cultured vascular smooth muscle cells [10].

Binding of the insurmountable antagonist [^{3}H]candesartan to intact CHO-hAT_1 cells has already been studied into some detail. This antagonist displayed high affinity binding to the human angiotensin AT_1 receptors. The binding was long-lasting with a half-life of about 2 h [7] and, as shown in the present study, almost none of it could be extracted from the CHO-hAT_1 cells upon mild acid treatment (Table 1). Previous studies have also pointed out that certain angiotensin AT_1 receptor antagonists display acid-resistant binding. Conchon *et al*. [14] found 30% of the [^{3}H]losartan binding and 80% for [^{125}I][Sar^1-Ile^8]angiotensin II binding to be acid-resistant in CHO cells transfected with rat AT_{1A} receptors. In the same line, 25% acid-resistance was found for [^{125}I][Sar^1-Ala^8]angiotensin II binding in cultured bo-

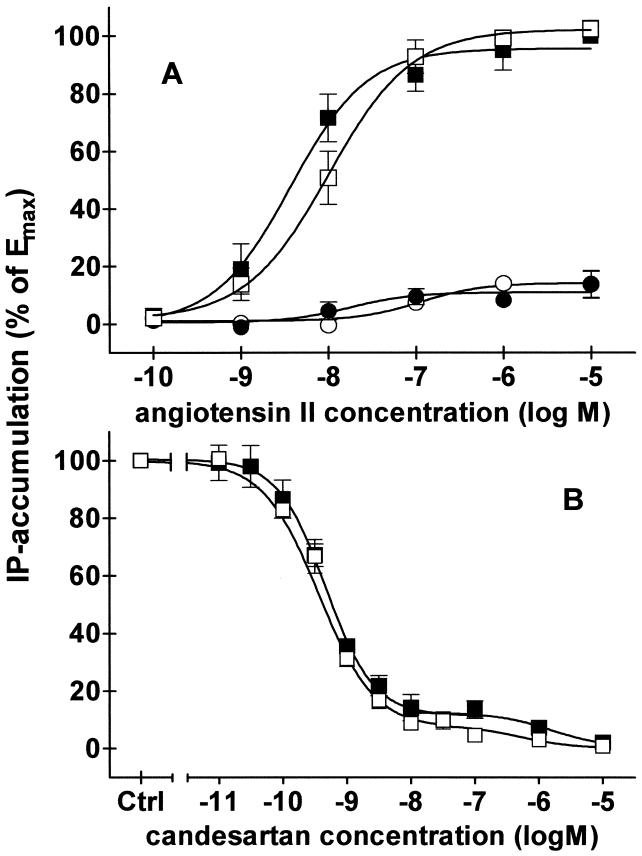

Fig. 7. Angiotensin II induced IP accumulation on both CHO-rAT_{1A}-WT and CHO-rAT_{1A}-TL_{314} cells. (A) represents the response on a 5 min incubation with increasing angiotensin II concentration on CHO-rAT_{1A}-WT (■) and CHO-rAT_{1A}-TL_{314} cells (□); EC_{50}'s are 3.7 ± 1.8 nM and 10.0 ± 4.0 nM respectively. Pre-incubation of 5.0 nM candesartan (30 min, 37°) decreased the maximal angiotensin II response on both CHO-rAT_{1A}-WT (●) and CHO-rAT_{1A}-TL_{314} cells (○) by 86 ± 4% and 89 ± 8% respectively. In (B), CHO-rAT_{1A}-WT (■) and CHO-rAT_{1A}-TL_{314} cells (□) have been pre-incubated for 30 min with increasing concentrations of candesartan and subsequently challenged with a 5 min incubation of 10 μM angiotensin II to induce IP accumulation. The data are mean values ± S.E.M. of 3 to 4 independent experiments, two determinations each and represented as % of the maximal inducible response at 10 μM angiotensin II for 5 min.

vine adrenocortical cells by Crozat *et al*. [11]. It was assumed that all [11], or at least some [14] of the acid-resistant binding accounted for internalized antagonist molecules and that this internalization occurred by receptor-mediated endocytosis.

In the present study, potential antagonist control of AT_1 receptor internalization was also investigated using additional criteria. Hypertonic sucrose has been found to abolish the internalization of β-adrenergic receptors via clathrin-coated pits. Its effect became a standard procedure to investigate the involvement of clathrin-coated pits in the internalization of other G-protein-coupled receptors [21–24]. In agreement with this general mechanism and with morphological studies showing the involvement of coated pits in the internalization of angiotensin II-angiotensin AT_1 receptor complexes [10], sucrose produced a dramatic reduction in the acid-resistant binding of [^{3}H]angiotensin II to the CHO-hAT_1 cells (Table 1). The concomitant rise in acid-sensitive binding of this agonist (Table 1) was to be expected since the receptors no longer disappeared from the cell surface by internalization. The internalization of [^{3}H]angiotensin II was almost completely inhibited by 0.4 M sucrose. This effect was rapid since it did not even require the cells to be preincubated with sucrose (Fig. 1a). In addition, sucrose provoked a time-dependent decrease in the cell-surface, acid-sensitive binding and total binding of [^{3}H]angiotensin II. The decrease occurred with a half-life of 20 to 30 min (Fig. 1a), which was substantially slower than the apparently immediate inhibitory effect on receptor internalization. This was not due to the detachment of cells from the multiwell plates since the total protein content remained unchanged after 90 min sucrose treatment. Sucrose also produced a time-wise reduction of the [^{3}H]candesartan binding to the intact CHO-hAT_1 cells with a half-life of about 90 min (Fig. 1). This reduction was even slower than the decline of cell surface [^{3}H]angiotensin II binding and therefore also unrelated to the ability of sucrose to prevent internalization. This effect was not associated to an increased viscosity of the medium since the association kinetics of the [^{3}H]candesartan to intact CHO-hAT_1 cells was not influenced by sucrose. Moreover, sucrose did not affect the candesartan and angiotensin II binding properties on CHO-hAT_1 cell membranes (Fig. 4 and Table 2). As a whole, these data indicated that sucrose merely affected the number of binding sites for both radioligands in time.

Interestingly, sucrose was found to produce a rapid decline in the [^{3}H]thymidine uptake in CHO-hAT_1 cells (Fig. 3). Because basal [^{3}H]thymidine uptake is often used as a marker for vital cellular functions [25–27], this decline indicated a severe effect of sucrose on cell viability. This could indirectly result in the decreased [^{3}H]angiotensin II and [^{3}H]candesartan binding on intact CHO-hAT_1 cells. However there was an apparent delay between the slow time-dependent reduction in [^{3}H]candesartan binding and the reduction in the cell surface [^{3}H]angiotensin II binding and the fast decrease in [^{3}H]thymidine uptake in the presence of 0.4 M sucrose (Fig. 1 and 3). It might be speculated that sucrose as a primary effect reduced cell viability and that subsequent effects like cell death and related processes such as receptor degradation, oxidation and liberation of proteases apparently resulted in the slower reduction in [^{3}H]angiotensin II and [^{3}H]candesartan binding.

As a second approach to investigate the potential link between angiotensin AT_1 receptor internalization via clathrin-coated pits and the tight binding of candesartan, use was made of CHO-rAT_{1A}-WT cells expressing wild type rat AT_{1A} receptors, and CHO-rAT_{1A}-TL_{314} cells expressing L^{314} truncated rat AT_{1A}-receptors with reduced internalization [16,28]. For the control CHO-rAT_{1A}-WT cells, the acid-resistant (internalized) [^{3}H]angiotensin II binding exceeded the acid-sensitive (cell surface) portion of the binding, but this situation was inverted for the CHO-rAT_{1A}-

TL_{314} cells (Fig. 6a,b). These findings are compatible with a decreased internalization of the truncated receptors and confirm those reported earlier by Thomas *et al.* [16]. Interestingly, the wild type and the truncated receptors displayed comparable [^{3}H]candesartan dissociation rate constants in binding studies and above all, preincubation of both cell lines with candesartan caused a similar, dramatic reduction of the maximal angiotensin II induced response (Fig. 7). These data indicated that candesartan still tightly bound to the vast majority of the truncated receptors in the CHO-rAT_{1A}-TL_{314} cells. Hence, the insurmountable behavior of candesartan is independent of receptor internalization. The distinction between the fast dissociating/surmountable state and the slow dissociating/insurmountable states of the antagonist-angiotensin AT_1 receptor complexes does therefore not reside at the level of sub-cellular receptor localization. In the same line, the angiotensin AT_1-specific antagonist PD 134756 did not affect the subcellular distribution of the AT_1 angiotensin receptors in direct visualization experiments by fluorescence labeling in human embryonic kidney 293 cells [10]. Moreover, the angiotensin II-mediated receptor internalization was even completely blocked in the presence of this antagonist.

The [^{3}H]candesartan binding to the CHO-rAT_{1A}-TL_{314} cells was found to be exclusively acid-resistant, despite the reduced receptor internalization in these cells (Fig. 6c,d). This did not differ from the binding to CHO-rAT_{1A}-WT and CHO-hAT_1 cells. Also no increased acid-sensitive [^{3}H]candesartan binding was seen on intact CHO-hAT_1 cells in the presence of sucrose (Table 1). The consistent absence of acid-sensitive [^{3}H]candesartan binding in these different experimental setups raised the question about the ability of the mild acid treatment to dissociate [^{3}H]candesartan from its receptor. This was directly evaluated on CHO-hAT_1 cell membranes. As shown in Fig. 5, exposure of these membranes to a mild acid buffer did not substantially dissociate [^{3}H]candesartan within the experimental period. This indicated that, unlike with [^{3}H]angiotensin II, mild acid treatment was unsuitable to discriminate between surface bound and internalized [^{3}H]candesartan.

One of the major findings in the present study is that [^{3}H]candesartan still tightly binds to the vast majority of the internalization deficient angiotensin rAT_{1A}-TL_{314} receptors. In agreement with the AT_1 angiotensin receptor visualization experiments by Hein *et al.* [10], the presented data rather suggest that non-peptide antagonist-angiotensin AT_1 receptor complexes remain at the cell surface. The present study also stresses that some of the criteria that were used previously to discriminate between internalized and cell surface-bound AT_1 receptor antagonists are unreliable for non-peptide AT_1 receptor antagonists. Mild acid treatment can discriminate between internalized and cell surface bound [^{3}H]angiotensin II, it cannot provide information with regard to the subcellular localization of bound [^{3}H]candesartan since this binding is resistant to mild acid. In addition, the sucrose sensitivity of AT_1 receptor agonist and antagonist ligand binding is not necessarily related to the inhibition of receptor internalization.

Acknowledgments

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