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Distinct binding properties of the AT_1 receptor antagonist [^{3}H]candesartan to intact cells and membrane preparations

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Abstract

[^{3}H]-2-Ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid ([^{3}H]candesartan), a non-peptide angiotensin II type 1 receptor (AT_1 receptor) antagonist bound with high affinity and specificity to intact adherent human AT_1 receptor transfected Chinese hamster ovary cells. The binding characteristics were preserved when cells were suspended, but the dissociation was 3–4-fold faster and the affinity 2-fold lower, while examining [^{3}H]candesartan binding to cell membranes. These data suggested the role of the intracellular organisation of living CHO-hAT_1 cells in antagonist–AT_1 receptor interactions. Yet, a specific role of microtubule or actin filaments of the cytoskeleton, receptor phosphorylation by Protein Kinase C, membrane polarity, cytoplasmic components like ATP and the need of an intact cell membrane could be excluded. The potential effect of protease degradation or receptor oxidation during the membrane preparation was also unlikely. The dissociation rate and the equilibrium dissociation constant of [^{3}H]candesartan increased with the temperature for both intact cells and membranes. Thermodynamic studies suggested that the bonds between candesartan and the hAT_1 receptor may be of different nature in intact CHO-hAT_1 cells and membranes thereof. Whereas the binding was almost completely enthalpy-driven on intact cells, there was a mixed contribution of both enthalpy and entropy on membranes.

Keywords: Angiotensin AT_1 receptor; Candesartan; Non-peptide antagonist; Dissociation; CHO cells; Membranes

1. Introduction

Angiotensin II, the major effector peptide of the renin–angiotensin system, mediates vascular smooth muscle cell contraction and growth, blood pressure regulation and aldosterone secretion by the G-protein-coupled angiotensin type 1 receptor (AT_1) [1,2]. The properties of AT_1 receptor antagonists are traditionally determined in '*in vitro*' contraction studies of isolated vascular smooth muscle cell preparations [3,4] and by radioligand-binding studies on isolated membrane preparations [5,6]. In contrast, Chinese hamster ovary cells stably expressing the transfected human AT_1 receptor (CHO-hAT_1 cells), allow both binding and functional studies to be performed under similar conditions [7]. In this respect, the non-peptide AT_1 receptor antagonist [^{3}H]candesartan was found to bind with high affinity to intact CHO-hAT_1 cells and to undergo slow dissociation from these cells ($t_{1/2} \sim 2$ hr) in the presence of a receptor-saturating concentration of unlabeled ligand (isotopic dilution) [8]. This coincided with the recovery of angiotensin II-mediated inositol phosphates accumulation in these cells. In a subsequent study, it appeared that the dissociation of [^{3}H]candesartan was about 3-fold faster in CHO-hAT_1 cell membranes as compared to the intact cells [9]. The aim of the present study is to find out the potential reasons for this discrepancy. The influence of membrane polarity, membrane integrity, energy depletion and elements of the cytoskeleton on the [^{3}H]candesartan dissociation rate from intact

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Abbreviations: AT_1 receptor, human angiotensin type 1 receptor; CHO-hAT_1 cells, Chinese hamster ovary cells expressing human AT_1 receptors; Candesartan, 2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid; Losartan, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)-biphenyl-4-yl)-methyl] imidazole; Irbesartan, 2-*n*-butyl-4-spirocyclopentane-1-[(2′-(1*H*-tetrazol-5-yl)-biphenyl-4-yl)-methyl]2-imidazolin-5-one.

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cells is addressed first. Thermodynamic properties of [^{3}H]candesartan to intact cells and membranes are compared next.

2. Materials and methods

2.1. Materials

Candesartan (CV-11974) [10] and losartan (DuP 753) [10] were obtained from AstraZeneca. Angiotensin II and BSA (Fraction V) were obtained from Sigma. [^{3}H]Cande-[^{3}H]Candesartan (22 Ci/mmol) was kindly provided by Takeda Chemical Industries Ltd. All other chemicals were of the highest grade commercially available.

2.2. Cells and preparations

CHO-hAT_1 cells were cultured in 75-cm^2 flasks in Dulbecco's modified essential medium that is supplemented with L-glutamine (2 mM), 2% of a stock solution containing 5000 IU/mL penicillin and 5000 μg/mL streptomycin (Life Technologies), 1% of a solution of Dulbecco's modified essential medium containing non-essential amino acids, 1 mM sodium pyruvate and 10% foetal bovine serum (Life Technologies) [7]. The cells were grown in 5% CO_2 at 37° until confluence. CHO-hAT_1 cell suspensions were obtained by harvesting the cultured cells *via* trypsinisation (0.05% trypsine, 0.02% EDTA in PBS) [9]. Control experiments revealed that the [^{3}H]candesartan binding was unaffected when cells are harvested by trypsinisation or scraping. The harvested cells were washed thrice at 4° by centrifugation (5 min at 500 *g*) and resuspension in fresh 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). To obtain membranes, the cell suspension was homogenised by Polytron and Potter homogeniser. The resulting homogenate was centrifuged (4°, 30 min at 27,000 *g*) and the pellet was resuspended in ice-cold HEPES buffer.

2.3. Radioligand binding

The binding of [^{3}H]candesartan to hAT_1 receptors on adherent intact CHO-hAT_1 cells in 24-well plates was measured as described previously [7,8]. At the start of the incubations, each well contained adherent CHO-hAT_1 cells (0.13 ± 0.03 mg protein content) and 400 μL HEPES buffer. Competition experiments were initiated by adding 50 μL buffer either alone (for total binding) or containing unlabeled competitor and 50 μL buffer containing 1.5 nM [^{3}H]candesartan (final concentration). Incubations were performed for 30 min at 37°. Kinetic experiments were started by adding 50 μL buffer containing 1.5 nM [^{3}H]can-[^{3}H]candesartan (final concentration) to each well and carried out at the indicated temperatures. Incubations lasted for 30 min (37°), 60 min (32°), 90 min (27°) and 180 min (22°). Dissociation of [^{3}H]candesartan was initiated by addition of 1 μM unlabeled candesartan. The incubations were stopped by washing the 24-well plates with 3 × 1.0 mL ice-cold HEPES buffer. The cells were then solubilised with 0.4 M NaOH and transferred to scintillation vials. A volume of 3.0 mL of scintillation liquid (Optisafe of Wallac) was added and the samples were counted for 3 min in a liquid scintillation counter.

Radioligand-binding assays on CHO-hAT_1 cell suspensions and membrane preparations thereof were performed as above but starting with 400 μL suspension (≈0.53 mg protein/mL) in glass tubes. The incubations were stopped by adding 3.0 mL ice-cold HEPES buffer. The membranes were subsequently filtered and washed on Whatmann's GF/B filters. Filters were placed in vials and counted as above.

In control experiments, a protease inhibitor cocktail (0.1 mM EDTA, 0.1 mM PMSF, 1 μM Pepstatin A, 1 μM Leupeptin) or different reducing agents (Vitamin C 10^{-4} M; sodium metabisulphite 10^{-4} M; or superoxide dismutase 1000 U/mL) were added after the first wash of the freshly harvested CHO-hAT_1 cells, and remained present during the experiment. Different treatments to assess the influence of the cellular integrity on [^{3}H]candesartan binding are listed in Table 1. For 24-hr pre-incubations, the drugs were already added in the culture medium.

2.4. Data treatment

Non-specific [^{3}H]candesartan binding (6 ± 1, 29 ± 9, and 32 ± 4% of total binding for plated cells, cell suspensions and membrane suspensions, respectively, for 1.5 nM [^{3}H]candesartan) was determined by adding 1 μM (final concentration) unlabeled candesartan to the samples just before adding the radioligand. The same values for non-specific binding were obtained with 1–10 μM unlabeled angiotensin II. For specific biding values, non-specific binding value was subtracted from total binding. The IC_{50} values from competition-binding experiments and the pseudo first order association rate constants from kinetic experiments (k_{obs}) were calculated by non-linear regression analysis using GraphPad Prism. Dissociation of [^{3}H]candesartan was exponential in all cases and rate constants (k_{-1}) were calculated by linear regression analysis of the logarithmic plot. The equilibrium dissociation constant of [^{3}H]candesartan was calculated from its kinetic constants ($K_d = k_{-1}/k_{+1}$). The K_i values of the competitors were calculated based on their IC_{50}'s, using the Cheng and Prusoff [11] equation: $K_i = IC_{50}\ (1 + K_d/[L])$. All competition curves were monophasic with Hill coefficients close to unity, indicating the presence of a homogeneous population of binding sites in each preparation. Thermodynamic parameters were derived from the van't Hoff's plots ($\ln(K_a)$ vs. $1/T$; $K_a = 1/K_d$ or $1/K_i$). For the calculations, the van't Hoff's plots were assumed to be linear and the equilibrium heat capacity difference (ΔC_p°) to be zero. The standard free Gibb's free energy (ΔG°), enthalpy

Table 1
Influence of different compounds on the [^{3}H]candesartan-, candesartan- and angiotensin II-binding affinities to hAT$_1$ receptors on intact CHO-hAT$_1$ cells in HEPES buffer at 37°

Compound (concentration, pre-incubation time)	Action	$T_{1/2}$ dissociation (mean ± SEM)	[^{3}H]Candesartan K_d (nM)	Angiotensin II K_i (nM)	Reference
Control		195 ± 33	0.021	0.13	
Colchicine (10 μM, 30 min)	Microtubulin depolarisation	348 ± 28	0.012	0.049	[12,13]
Vinblastine (10 μM, 30 min)	Free tubulin depletion	325 ± 23	0.013	0.059	[14]
Paclitaxel (10 μM, 24 hr)	Microtubulin stabilisation	318 ± 23	0.012	0.069	[13,15]
Mevastatin (10 μM, 24 hr)	Inhibition of mevolanate synthesis	246 ± 19	0.017	0.089	[16,17]
Cytochalasin B (1 μM, 24 hr)	Actin filament destabilisation	277 ± 9	0.013	0.071	[16,18]
Cytochalasin D (1 μM, 24 hr)	Actin filament destabilisation	251 ± 13	0.017	0.089	[16,18]
NaN_3 (1 mM, 60 min)	Inhibition of ATP-synthase	170 ± 12	0.033	0.166	[19]
GF109203X (1 μM, 30 min)	PKC inhibition	206 ± 12	0.018	0.174	[20,21]
KCl (0.1 mM, 15 min)	Membrane depolarisation	158 ± 32	0.024	0.126	
Saponine (0.001%)	Membrane permeabilisation	223 ± 42	0.022	0.060	

(ΔH°) and entropy (ΔS°) were calculated using following equations; $\Delta G^\circ = RT \ln(K_d)$ and $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$.

3. Results

The binding of 1.5 nM [^{3}H]candesartan to hAT$_1$ receptors reached equilibrium after 30 min at 37° for all preparations. Its dissociation rate was closely the same for adherent and suspended CHO-hAT$_1$ cells but about 3.4-fold faster for the membrane preparation (Fig. 1, Table 2). For the equilibrium dissociation constants, the K_d of [^{3}H]candesartan (calculated from kinetic data) and the K_i values of candesartan and losartan (calculated from competition binding IC_{50} values) were found to be nearly the same for adherent and suspended cells but to increase 2–4.5-fold for the membranes (Table 2).

To check the potential influence of proteolytic degradation and oxidation, a protease inhibitor cocktail or different antioxidants/reducing agents were added after the first wash of the freshly harvested CHO-hAT$_1$ cells, and remained present during the ensuing preparations and binding experiments. Yet, none of these treatments affected the [^{3}H]candesartan dissociation rate and antagonist equilibrium dissociation constants for suspended cells (as control) and membranes (Table 2 and data not shown for the antioxidants). To investigate the influence of the cytoskeleton, cellular physiology and/or integrity on the binding properties, adherent CHO-hAT$_1$ cells were pre-treated with the compounds listed in Table 1 and binding assays were performed in the continuing presence of each compound. Except for those compounds that influenced the microtubular structure, neither of these treatments significantly affected the dissociation rate and K_d of [^{3}H]candesartan, nor the K_i of angiotensin II (Table 1).

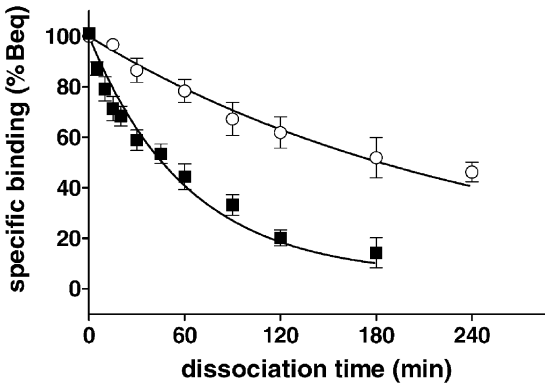

Fig. 1. Dissociation of [^{3}H]candesartan from the hAT$_1$ receptor under different conditions. Half-life on membranes: (■) 58 ± 5 min, on intact cells; and (○) 195 ± 27 min. Values are represented as mean ± SEM of 6–8 independent experiments.

The rates of [^{3}H]candesartan association and dissociation from adherent cells and membranes increased with temperature within the tested 22–42° range (Fig. 2). Lower temperatures were not investigated because of the extremely slow dissociation of the radioligand. The temperature dependence of the affinity of [^{3}H]candesartan (with $K_a = 1/K_d$) for both preparations is illustrated by van't

Table 2
Kinetic parameters and calculated K_d of the [^{3}H]candesartan binding to hAT$_1$ receptors on different preparations in HEPES buffer at 37°

	Intact cells	Suspension	Suspension + prot. inhibition	Membranes	Membranes + prot. inhibition
k_{-1}, ($\times 10^{-3}$ min^{-1})	3.55 ± 0.57	5.7 ± 0.8	4.8 ± 0.7	12.0 ± 1.2	14.4 ± 0.6
K_d, [^{3}H]candesartan (nM)	0.029	0.019	0.028	0.055	0.089
K_i, candesartan (nM)	0.040	0.024	0.039	0.181	0.157
K_i, losartan (nM)	3.45	1.54	2.01	5.76	8.67

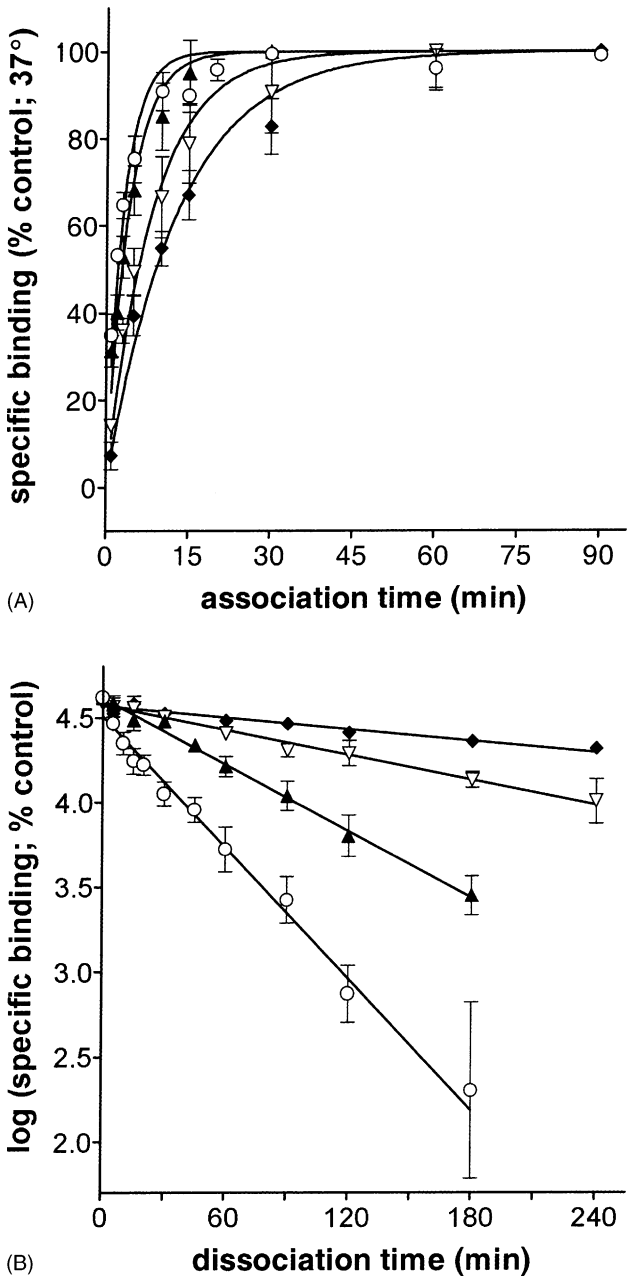

Fig. 2. [^{3}H]Candesartan binding; association (A) and dissociation (B) kinetics on membranes at different temperatures, i.e. 37 (○); 32 (■); 27 (▽); and 22 (◆).

Hoff's plots in Fig. 3. Because of the linearity of the plots, the equilibrium heat capacity difference (ΔC_p°) was set to zero in ensuing calculations. The [^{3}H]cande-^{3}H]candesartan binding was exothermic (i.e. the affinity increased with temperature) in both preparations; it was largely enthalpy-driven for adherent cells whereas for the membranes there was a mixed enthalpy (≈60%)–entropy contribution (Fig. 3). Thermodynamic parameters of losartan for adherent cells were calculated from competition-binding data at 37 and 27°. Losartan binding was also exothermic and essentially enthalpy-driven with a minor entropy contribution (Fig. 3).

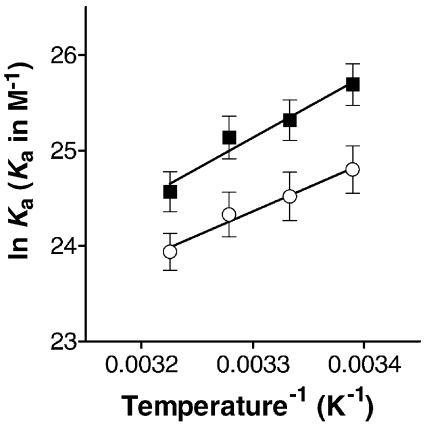

Fig. 3. van't Hoff's plot: thermodynamic characteristics of the [^{3}H]can-^{3}H]candesartan binding on membranes (○), and intact CHO-hAT_1 cells (■) derived from kinetic experiments at different temperatures in HEPES buffer. The values of the enthalpy (ΔH°) and entropy ($-T\,\Delta S^\circ$) at $T = 298.15$ K are, respectively, −42.1 and 19.7 kJ mol^{-1} for the [^{3}H]candesartan binding on membranes, −53.9 and –9.6 kJ mol^{-1} for the [^{3}H]candesartan binding on intact cells and −44.7 and −8.0 kJ mol^{-1} for the losartan binding on intact cells.

4. Discussion

During the last decade, much effort has been spend in developing non-peptide AT_1 receptor antagonists for the clinical treatment of hypertension and congestive heart failure. In pre-clinical studies, these antagonists are routinely tested for their ability to affect AII dose–contractile-response curves of vascular tissues such as rabbit aortic strips and of rat portal veins. Based on their different capabilities to depress the maximal contractile response to angiotensin II, the antagonists are commonly divided in surmountable ones (who don't produce a depression; typical example is losartan) and insurmountable ones. The extent of the depression by the insurmountable antagonists is highly variable; it is almost complete for candesartan but only partial for irbesartan, valsartan and EXP3174 (the active metabolite of losartan) [7,22–24]. Several theories have been put forward to explain the differences in behaviour of the AT_1 receptor antagonists in the past. Yet, recent studies on stably transfected CHO-cells that express the hAT_1 receptor (CHO-hAT_1 cells) have clearly established that they are competitive with angiotensin II and that their insurmountable behaviour is related to their ability to form tight-binding complexes with the receptor, i.e. the blockade is sufficiently long-lasting to prevent the access of the receptors to subsequently added angiotensin II [7,8].

To explain the partial nature of insurmountable inhibition, a model was recently put forth in which a antagonist–AT_1 receptor complexes may adopt fast and slow dissociating states, with only the latter being responsible for insurmountable antagonism [25]. The extent of this phenomenon is dependent of the equilibrium between both states (i.e. the proportion of occupied receptors residing in the tight-binding state) and this equilibrium appears to

depend on the stability/antagonist dissociation rate of the tight-binding state [26,27]. Except that tight-binding antagonist–AT_1 receptor complexes are not created by the internalisation of the receptor [9,28], it remains unclear what distinguishes them from the loose-binding state at the molecular level. In this context, it was surprising that the dissociation of the insurmountable antagonist [^{3}H]candesartan from membranes prepared from CHO-hAT_1 cells was appreciably faster as compared to the intact, adherent cells [8]. In the present study, it was shown that the binding parameters of [^{3}H]candesartan are not affected by suspending the cells but that preparation of the membranes increases its dissociation rate and decreased its binding affinity (Fig. 1, Table 2). Perturbation of proteins involved in cell adhesion, like adhesins and integrins, therefore, cannot account for the faster dissociation of [^{3}H]candesartan from membranes. This change was also unlikely to result from proteolytic degradation or oxidative phenomena since a protease inhibitor cocktail or different antioxidants/reducing agents were without effect (Table 2 and data not shown). The [^{3}H]candesartan dissociation rate on CHO-hAT_1 cell membranes agrees with the value established by Ojima et al. [6] on bovine adrenal cortical membranes ($t_{1/2} = 66 \pm 3$ min), which was also faster than the slow-recovery behaviour observed on rabbit aorta (intact cell system) after candesartan pre-treatment.

The CHO-hAT_1 cells are obviously disrupted during the membrane preparation. The faster dissociation of [^{3}H]can-^{3}H]candesartan from such membranes therefore suggests that the intracellular organisation of live CHO-hAT_1 cells may play a specific role in strengthening antagonist–AT_1 receptor interactions. In this context, it has been shown that the hAT_1 receptor like other GPCRs, interacts with the cytoskeleton. Besides the rapid cytoskeletal reorganisation that is required for the agonist-mediated internalisation of hAT_1 receptors *via* clathrin-coated pits [30,31], elements of the cytoskeleton, like tubulin and microtubuli also participate in the function and desensitisation of the receptor [12,29,31–34]. To get more insight on this issue, the influence of drugs that disrupt the cytoskeleton, cellular physiology and/or integrity, was subsequently investigated on [^{3}H]candesartan binding to adherent cells. However, compounds with the reputation to inhibit receptor phosphorylation by PKC, to deplete ATP levels or to disturb or stabilise microfilament structures of the cytoskeleton did not have a noticeable influence on the [^{3}H]candesartan and angiotensin II binding (Table 1). Moreover, abolishing the membrane polarity and other chemical gradients regulated by the cell membrane by increasing the extracellular potassium concentration or by cell permeabilisation, did not affect binding either (Table 1). As a consequence, none of these structures, proteins or gradients appear to be involved in the tight antagonist-binding state of the receptor. Intriguingly, [^{3}H]candesartan binding was noticed to be stronger in the presence of compounds that affect microtubular structures, regardless of their stability or destabilitory action. At the present level of investigation, as the effect is relatively weak, it is premature to speculate about the underlying mechanisms.

Determination of thermodynamic parameters of the [^{3}H]candesartan binding on intact cells and membranes might also give indications about the molecular mechanisms involved in the tight binding. The binding of [^{3}H]can-^{3}H]candesartan was exclusively enthalpy-driven on intact cells (Fig. 3). In the same vein, the binding of other non-peptide AT_1 antagonists of the biphenyltetrazole class like [^{3}H]irbesartan [35] and losartan (see legend of Fig. 3) to these cells is also mainly enthalpy-driven with a small entropy contribution. This agrees with the increasing evidence that ionic interactions play a major role in non-peptide antagonist–AT_1 receptor interactions [36]. Since losartan displays only loose binding to the hAT_1 receptor, it might be speculated that the formation of such complexes is mainly enthalpy-driven (-41 kJ mol^{-1}) for all antagonists. In this respect, enthalpy-driven ligand–receptor interactions are generally considered to be related to a conformational change that brings the ligand–receptor complex to a lower energetic level [37,38]. The larger enthalpy contribution for the insurmountable antagonist candesartan as compared to losartan (Fig. 3) may, therefore, reflect the conversion of the antagonist–receptor complexes to a tighter binding state.

In contrast, [^{3}H]candesartan binding to cell membranes displayed a mixed enthalpy–entropy-driven contribution with an entropy contribution of about 40% (Fig. 3). This type of antagonist behaviour has previously also been observed for several other transmembrane receptors like dopamine D2, serotonin 5-HT_3 and adenosine A_1 and A_{2a} receptors [39,40]. Based on studies with β-adrenergic receptors, it was initially hypothesised that agonist binding is enthalpy-driven, while antagonist binding is entropy-driven [38]. The more recent findings now clearly indicate that this hypothesis should not be generalised.

Due to the lower enthalpy contribution of the binding, it is plausible that candesartan–AT_1 receptor complexes are no longer able to adopt the same tight-binding conformation as in the intact CHO-hAT_1 cells. Since an increase in entropy is considered to be due to solvent reorganization like the water molecules around the ligand and its binding site [37,38], our data also suggest that the bonds between candesartan and the hAT_1 receptor may be of different nature in intact CHO-hAT_1 cells and membranes thereof.

To summarise, the intracellular organisation of living CHO-hAT_1 cells may play a specific role in strengthening the interaction between insurmountable antagonists like candesartan and AT_1 receptors. Yet, the exact molecular mechanism remains to be established. Differences in antagonist-binding profiles between intact cells and derived membrane preparations is not unique for the AT_1 receptor system, because, recently it has also been observed for endothelin ET_b receptors [41]. Because of these two findings it should be of interest to investigate

whether similar differences in ligand affinity also take place for other receptor systems.

Thermodynamic characterization revealed that [^{3}H]candesartan binding was almost completely enthalpy-driven interaction for intact cells as compared to a mixed contribution of both enthalpy and entropy for cell membranes. Noteworthy is the strong temperature sensitivity of the binding of [^{3}H]candesartan and other AT_1 receptor antagonists like [^{3}H]irbesartan [35]. These findings stress the importance of the same experimental set-up (receptor preparation, temperature, ...) when comparing kinetic, affinity and inhibition data.

Acknowledgments

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