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Biochemical Pharmacology 67 (2004) 1601–1606
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Effect of saponin and filipin on antagonist binding to AT_1 receptors in intact cells

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Received 27 October 2003; accepted 12 January 2004

Abstract

In the present study, [^{3}H]-candesartan binding experiments were performed on intact Chinese Hamster Ovary cells transfected with the human AT_1 receptor (CHO-AT_1 cells). Cells were pre-treated with 0.01 mg/ml saponin or filipin. Both pre-treatments resulted in an increased dissociation rate and decreased affinity of the insurmountable non-peptide antagonist [^{3}H]-candesartan. A similar decrease in affinity was observed for the peptide antagonist Sar^1-Ile^8 angiotensin II and for other non-peptide antagonists, irrespectively of their degree of insurmountability. A similar discrepancy in [^{3}H]-candesartan binding was earlier observed when comparing intact CHO-AT_1 cells and membrane preparations thereof. This similarity is further highlighted by the observations that saponin or filipin no longer affect [^{3}H]-candesartan binding to CHO-AT_1 cell membranes and that both agents permeabilise the CHO-AT_1 cells. This suggests that the intracellular composition and/or organisation of living cells play an active role with regard to antagonist–AT_1 receptor interactions.

Keywords: Angiotensin II; AT_1 receptor; Antagonist; Filipin; Saponin; Permeabilisation

1. Introduction

Angiotensin II (Ang II), the major effector protein of the renin angiotensin system, mediates its hypertensive effects by the G-protein coupled angiotensin type 1 (AT_1) receptor. Several peptide and non-peptide AT_1 receptor antagonists have been developed. Recent studies with intact Chinese Hamster Ovary cells stably expressing the human AT_1 receptor (CHO-AT_1 cells) revealed that such antagonists are competitive with Ang II [1]. However, the antagonists show marked differences when considering their ability to adopt fast reversible and tight binding complexes with the AT_1 receptor [2]. Whereas losartan, the prototype of the non-peptide antagonists, is only able to form fast reversible complexes, others like candesartan almost exclusively form tight binding complexes. In the presence of non-peptide antagonists like valsartan and the peptide antagonist Sar^1-Ile^8 angiotensin II, both types of complexes appear to coexist in almost equal proportions. This may explain the partial nature of the insurmountable behaviour of such antagonists in functional studies. In accordance with the formation of tight binding complexes, [^{3}H]-candesartan was found to bind with high affinity to intact CHO-AT_1 cells and to dissociate slowly from those cells [3].

Fierens et al. [4] recently also reported that the dissociation of [^{3}H]-candesartan from isolated CHO-AT_1 cell membranes was about 3.4 times faster when compared to the parent intact cells. Yet, no apparent cause for this difference could be established [4]. In this respect, it is of interest that the fluidity and lipid composition of isolated membranes may differ from those in intact cells [5,6]. Whereas intrinsic membrane proteins like G protein coupled receptors (GPCR) were originally regarded to float in a homogeneous sea of lipids, the existence of cholesterol-enriched membrane microdomains like lipid rafts and caveolae is now well recognised. These microdomains are less fluid than the surrounding plasma membrane and cholesterol appears to be essential in maintaining their structural integrity [7–10]. Evidence is

Abbreviations: Ang II, angiotensin II; AT_1, angiotensin II receptor type 1; CHO-hAT_1 cells, Chinese Hamster Ovary cells expressing human AT_1 receptors; GPCR, G-protein coupled receptor; SAM, *S*-adenosylmethionine.

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doi:10.1016/j.bcp.2004.01.004

now also accumulating for the role of these microdomains in controlling the function of receptors and other membrane proteins [11].

These considerations prompted us to further investigate antagonist–AT_1 receptor interactions in relation to the membrane integrity. Structural disorder of the membrane was obtained with the cholesterol-binding agents saponin and filipin. Saponin is especially known for its capability to form membrane pores [12] whereas filipin is able to sequester cholesterol in the membrane [13,14].

2. Materials and methods

2.1. Materials

Candesartan (CV-11974; 2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid), EXP 3174 (2-*n*-butyl-4-chloro-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid), losartan (DuP 753; 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole) and the tritiated ligand [^{3}H]-candesartan (16 Ci/mmol) were obtained from AstraZeneca (Mölndal, Sweden). Valsartan ((*S*)-*N*-valeryl-*N*-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-valine was obtained from Novartis (Basel, Switzerland) and Sar^1-Ile^8 Angiotensin II was obtained from Neosystem (Strasbourg, France). All other chemicals were of the highest grade commercially available.

2.2. Cell culture

Chinese Hamster Ovary cells stably expressing the human angiotensin II AT_1 receptor (CHO-AT_1 cells [15]) were cultured in 75 cm^2 flasks in Dulbecco's modified essential medium supplemented with L-glutamine (2 mM), 2% of a stock solution containing 5000 IU/ml penicillin and 5000 μg/ml streptomycin (Invitrogen), 1% (v/v) of a solution of MEM containing non-essential amino acids, 1 mM sodium pyruvate, and 10% (v/v) fetal calf's serum (Invitrogen). The cells were subsequently grown in 24 well plates. CHO-AT_1 cell membranes were prepared as described in Vanderheyden et al. [15].

2.3. [^{3}H]-Candesartan binding

Before the experiment, the plated cells were washed twice with HEPES buffer (137 mM NaCl, 20 mM HEPES, 2.7 mM KCl, 1.8 mM $CaCl_2$, 2.1 mM $MgCl_2$, pH 7.4) at room temperature (0.5 ml per well) and then incubated with HEPES buffer for 10 min at 37 °C. When appropriate, cells were first incubated with 0.5 mM *S*-adenosylmethionine (SAM) for 30 min at 37 °C and the compound remained present throughout the experiment. For all experiments, cells were incubated for 10 min with HEPES buffer alone (control) or with buffer containing 0.01 mg/ml saponin, 0.01 mg/ml filipin. These compounds remained present throughout the ensuing binding experiments.

All binding experiments were performed in a final volume of 0.5 ml. In association experiments the cells were incubated for the given time periods at 37 °C with 1.5 nM [^{3}H]-candesartan. For dissociation experiments, cells were incubated with 3 nM radioligand for 40 min at 37 °C. One micromolar unlabelled candesartan was added and cells were further incubated for the indicated time periods. Competition binding experiments were performed by incubating the cells for 60 min at 37 °C with 1.5 nM [^{3}H]-candesartan in the presence of increasing concentrations of unlabelled antagonists. For saturation binding assays, CHO-AT_1 cells were incubated for 60 min at 37 °C with increasing concentrations of [^{3}H]-candesartan (0.03 and 4.9 nM). Non-specific [^{3}H]-candesartan binding was measured in the presence of 1 μM unlabeled candesartan. At the end of each incubation, the cells were briefly washed three times with HEPES buffer at 4 °C. The cell bound radioactivity in each well was subsequently solubilised with 500 μl sodium hydroxide (0.2 M) and counted for 3 min in a liquid scintillation counter after addition of 3 ml scintillation liquid (Optisafe, Perkin-Elmer).

Radioligand binding assays on CHO-hAT_1 cell membranes were performed as above but were carried out in glass tubes. The incubations were stopped by adding 3.0 ml ice-cold HEPES-buffer and the membranes were subsequently filtered and washed on Whatmann GF/B filters. Filters were placed in vials and counted as above.

2.4. Corrections and calculations

Saponin produced a slow time-wise loss in the number of attached cells. Whereas this loss was only minimal in association experiments, it needed to be accounted for in the much longer dissociation experiments. For this purpose, radioligand binding in the absence of unlabeled candesartan was taken as the control (i.e. 100%) binding at each time point. Filipin did not cause prominent cell loss in the different experiments.

Non-specific [^{3}H]-candesartan binding was subtracted from the total binding to yield specific binding. The calculation of the parameters from the dissociation curves (k_{-1}), association curves (k_{obs}), saturation binding curves (K_D) and competition curves (IC_{50}) was performed by non-linear regression analysis using GraphPad Prism using monoexponential equations, i.e.: association: $Y = Y_{max}(1 - e^{(k_{obs} \cdot X)})$; dissociation: $Y = 100\, e^{(-k_{-1} \cdot X)}$; saturation: $Y = B_{max} \cdot X/(K_D + X)$; competition: $Y = 100/(1 + 10^{(X - \log(IC_{50}))})$. Data points were the mean and S.E.M. of at least three separate experiments with triplicate determinations each. The second order association constant (k_{+1}) equals ($k_{obs} - k_{-1}$)/[radioligand concentration, L]. The k_{-1}/k_{+1} ratio provides an alternative estimation of the

[^{3}H]-candesartan equilibrium dissociation constant (kinetic K_D). The K_i values of the competitions are calculated from their IC_{50}s, using the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + K_D/[L])$, where K_D = kinetic K_D.

2.5. Trypan blue staining

Cells were washed and incubated from 10 up to 120 min at 37 °C with HEPES buffer, with or without 0.01 mg/ml saponin or filipin. Trypan blue (0.4%) was added and after 7 min cells were three times washed with HEPES buffer.

3. Results

Binding of 1.5 nM [^{3}H]-candesartan to CHO-hAT$_1$ cells increased time-dependently and reached equilibrium within 30-min incubation at 37 °C. The corresponding pseudo first-order association rate constant (k_{obs}) was 0.18 ± 0.02 min^{-1}. The dissociation occurred exponentially, with a k_{-1} of 0.0035 ± 0.0004 min^{-1} (Table 1).

Treatment of the cells with saponin or filipin (0.01 mg/ml) slowed-down the association (±3-fold decrease in k_{obs}; Fig. 1) and accelerated the dissociation (four- to seven-fold increase in k_{-1}; Table 1; Fig. 2A). The calculated k_1 values were four- to six-fold higher and, when calculated as k_{-1}/k_{+1}, the K_D of [^{3}H]-candesartan was ±20–40 times higher for the treated cells (Table 1).

For saponin- and filipin-treated cells, there is good agreement between the 'kinetic' and 'thermodynamic' K_D values (Table 1). No such comparison can be made for control cells because of the very high affinity of [^{3}H]-candesartan hampers a correct estimation of the 'thermodynamic' K_D value [3,16].

Competition binding curves of losartan, valsartan and Sar^1-Ile^8 angiotensin II are monophasic for control, saponin- and filipin-treated cells (data not shown). As for the radioligand, the K_i values of these antagonists are all increased by 10–20 times for the treated cells (Table 2). Consequently, the potency ratio of the tested antagonists is not affected, i.e. losartan < valsartan < Sar^1-Ile^8 angiotensin II.

Pre-treatment of the cells with *S*-adenosylmethionine (SAM) was unable to prevent the accelerating effect of saponin and filipin on the [^{3}H]-candesartan dissociation.

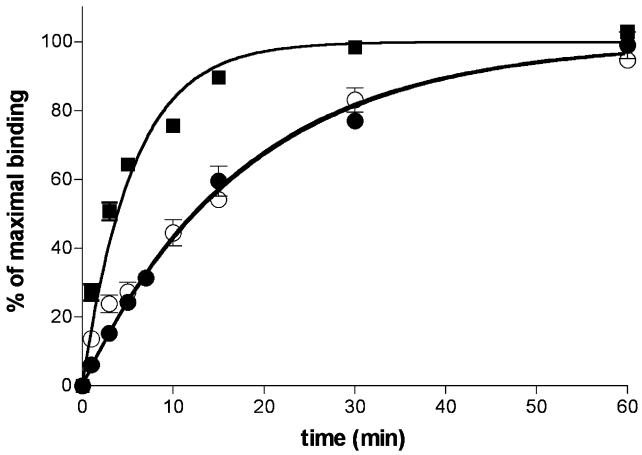

Fig. 1. Time course of specific binding of 1.5 nM [^{3}H]-candesartan to CHO-AT$_1$ cells after pre-incubation for 10 min at 37 °C with buffer (■), 0.01 mg/ml saponin (○), or 0.01 mg/ml filipin (●). Maximal binding was calculated by non-linear regression analysis with GraphPad Prism using monoexponential equations.

Table 2
Binding parameters of unlabeled antagonists obtained from competition binding with 1.5 nM [^{3}H]-candesartan on CHO-AT$_1$ cells calculated from $K_i = IC_{50}/(1 + K_D/[L])$

	Buffer	Saponine	Filipin
IC_{50} (nM)			
Losartan	147.0 ± 42.5	101.8 ± 17.9	218.0 ± 27.8
Valsartan	15.9 ± 0.4	18.3 ± 0.5	29.1 ± 4.4
Sar^1-Ile^8 Ang II	9.6 ± 2.0	10.4 ± 2.0	18.8 ± 2.7
K_i (nM)			
Losartan	2.85 ± 0.83	27.5 ± 4.8	52.2 ± 11.7
Valsartan	0.31 ± 0.01	4.95 ± 0.14	7.28 ± 1.26
Sar^1-Ile^8 Ang II	0.19 ± 0.04	2.82 ± 2.0	5.36 ± 0.77

Calculated k_{-1} values (0.0141 ± 0.0013 min^{-1} and 0.0102 ± 0.0018 min^{-1}, respectively) are similar to those in the absence of SAM (Table 1). The dissociation of [^{3}H]-candesartan is appreciable faster from isolated CHO-hAT$_1$ cell membranes as compared to the intact cells (Fig. 2B) but there is no longer an additional accelerating effect of saponin or filipin either alone or in combination with SAM (data not shown).

Trypan blue staining reveals that 0.01 mg/ml saponin and filipin are able to permeabilise intact CHO-hAT$_1$ cells. Full permeabilisation is already achieved after 10 min with filipin and after 30 min with saponin (Fig. 3).

Table 1
Kinetic parameters of [^{3}H]-candesartan binding on CHO-AT$_1$ cells

	Control	Saponin	Filipin
k_{obs} (min^{-1})	0.18 ± 0.02	0.057 ± 0.006	0.056 ± 0.004
k_{+1} (min^{-1} nM^{-1})	0.118 ± 0.011	0.028 ± 0.004	0.020 ± 0.003
k_{-1} (min^{-1})	0.0035 ± 0.0004	0.0152 ± 0.0021	0.0264 ± 0.0014
Kinetic K_D	0.03	0.55	1.3
K_D (saturation binding)	0.086 ± 0.003*	0.57 ± 0.14	0.59 ± 0.23

* This value is overestimated ([3,16]).

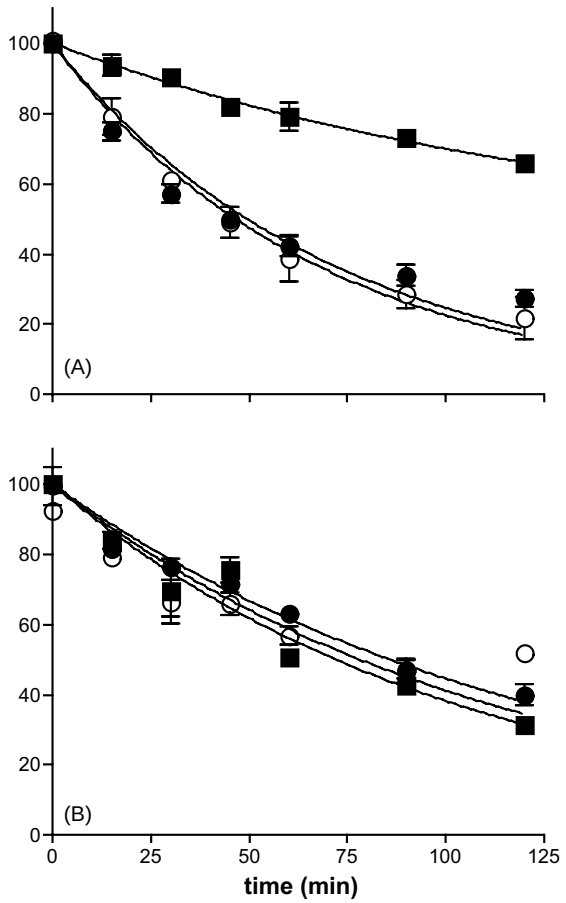

Fig. 2. Dissociation of 3 nM [^{3}H]-candesartan from intact CHO-AT_1 cells (A) and membranes (B) after pre-incubation for 10 min at 37 °C with buffer (■), 0.01 mg/ml saponin (○), or 0.01 mg/ml filipin (●).

4. Discussion

In the present study, it is shown that the cholesterol-modulating agents saponin and filipin cause a marked decrease in the affinity of different AT_1 receptor antagonists for human AT_1 receptors in CHO cells. This effect occurs at low concentrations of both agents (0.01 mg/ml) and only in intact cells. It is independent from the antagonist's capacity to induce insurmountable binding and appears to be related to cell permeabilisation rather than to disrupting the membrane-stabilising function of cholesterol.

Previous studies have shed light on the ability of antagonist–AT_1 receptor complexes to adopt two distinct states: a fast reversible state which is responsible for surmountable inhibition, and a tight binding state for insurmountable inhibition. A two-step, two-state model provides a simple and adequate description of this comportment: the antagonist (I) first undergoes a fast reversible binding to the AT_1 receptor (R) and the complex (IR) is then able to undergo a further conversion into a tight binding state (IR^*).

$$I + R \Leftrightarrow IR \Leftrightarrow IR^*$$

The degree of insurmountability, defined as the IR^*/IR ratio, is specific for each antagonist and is largely dictated by the stability of IR^* [17]. This adequately explains why the slowly dissociating antagonist [^{3}H]-candesartan ($t_{1/2}$ ~120 min in intact CHO-hAT_1 cells) is almost exclusively insurmountable in functional studies. Since saponin and filipin produced a net decrease in the dissociation rate (four- to seven-fold) and affinity of [^{3}H]-candesartan for the AT_1 receptors in intact CHO-hAT_1 cells, it could be argued that these agents interfere with the formation of IR^*. A previous receptor mutation study by Fierens et al. [18] reveals that such interference should decrease the affinity of antagonists to an extent that is proportional to their degree of insurmountability [4]. However, in this study, saponin and filipin produced a comparable decrease in affinity of all non-peptide antagonists, regardless of their surmountable (losartan), partial insurmountable (valsartan) or almost complete insurmountable (candesartan) nature. Moreover, despite the quite different structure of the peptide antagonist Sar^1-Ile^8 angiotensin II, both agents also decreased its affinity. These findings suggest that saponin and filipin affect the overall receptor conformation rather than specific regions, such as Lys^{199}, that are implicated in the stability of IR^* [18].

The influence of membrane cholesterol on the conformational state of intrinsic proteins like GPCRs has already been evoked in various studies. In this respect, it is known that cholesterol-modulating agents like saponin and filipin produce a decrease in membrane fluidity [12,19] and that

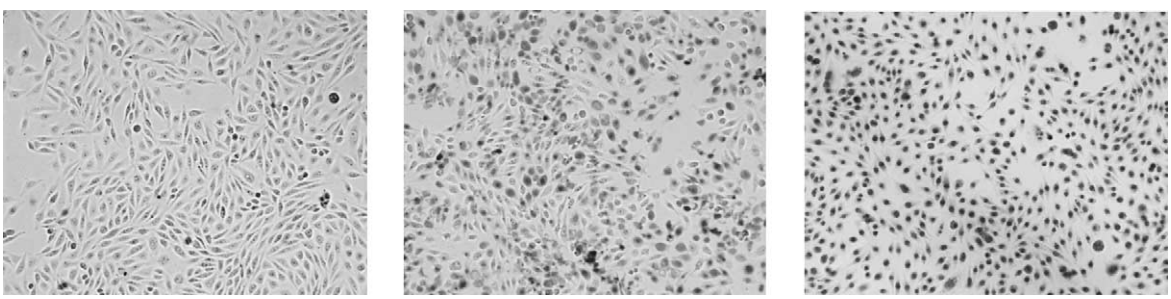

Fig. 3. Trypan blue staining: CHO-AT_1 cells were stained after 30-min incubation at 37 °C with buffer (left), 0.01 mg/ml saponin (middle), or 0.01 mg/ml filipin (right). Cells with dark (coloured) nucleus or cytoplasm are permeabilised.

they interfere with receptor signalling [9,20–23]. On the other hand, SAM increases the membrane fluidity by increasing the conversion of phosphatidylethonalamine to phosphatidylcholine [24,25] and this has been associated with an increased activity of GPCRs like α1-adrenergic receptors in intact cells [21]. However, treatment of the CHO-hAT_1 cells with SAM did not affect the dissociation rate of [^{3}H]-candesartan nor the effect of saponin of filipin thereon (data not shown). It is, therefore, unlikely that the latter agents decreased affinity of the AT_1 receptor antagonists by merely decreasing the membrane fluidity.

In the past decade, evidence has grown that the plasma membrane is organised in microdomains. Among those, lipid rafts are localised regions with elevated cholesterol and glycosphingolipid levels [11]. These are less fluid than the surrounding plasma membrane and cholesterol contributes to their stability. Caveolae form a subset of lipid rafts; they are characterised by the presence of the cholesterol-binding protein caveolin-1. A large number of GPCRs have been shown to be enriched in lipid rafts and caveolae [11] and initial findings suggested that activated AT_1 receptors also accumulate in caveolae [26]. Yet, more recent receptor internalisation studies and electron microscopic visualisation studies failed to confirm this process and even indicate that AT_1 receptors are almost evenly distributed among lipid rafts and cholesterol-independent microdomains [27,28].

Although depletion of cellular cholesterol with filipin will remove cholesterol from lipid rafts and eventually lead to their disruption [29–36], two observations plead against the implication of this process in the altered antagonist binding properties of the AT_1 receptor. First, a uniform alteration of antagonist binding properties, as observed in the present study, is not compatible with a heterogeneous distribution of the receptor among lipid rafts and cholesterol-independent microdomains [28]. Next, the failure of saponin and filipin to affect the [^{3}H]-candesartan binding properties in CHO-hAT_1 cell membrane preparations does not fit with the persistence of lipid rafts in such preparations [11]. In this respect, it is of interest that the treatment of intact cells with saponin and filipin produces their permeabilisation (Fig. 3) and that the dissociation of [^{3}H]-candesartan is affected in the same way as the process of membrane preparation [4]. Therefore, we propose that the effect of both agents on antagonist–AT_1-receptor interactions result in a large extent from their propensity to permeabilise the intact CHO-hAT_1 cells. This suggests that the intracellular composition and/or organisation of living cells play an active role with regard to antagonist–AT_1 receptor interactions and, seen the similar effect for endothelin ET_b receptors [37], presumably for antagonist-GPCR interactions in general. This role still remains to be disclosed but microtubili or actin filaments, receptor phosphorylation, membrane polarity and cytoplasmic components like ATP are unlikely to be involved [18].

Acknowledgments

We are obliged to the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen, the Vlaams Instituut voor de bevording van het Wetenschappelijk Technologisch Onderzoek in de Industrie (IWT) and AstraZeneca, Sweden for their financial support.

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