Inhibition of Angiotensin II-Induced Inositol Phosphate Production by Triacid Nonpeptide Antagonists in CHO Cells Expressing Human AT_1 Receptors

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Purpose. The aim of the present work is to describe the inhibitory properties of LY301875 and LY303336, two polysubstituted 4-aminoimidazole AT_1 receptor antagonists, on CHO cells expressing human recombinant AT_1 receptors.

Methods. The binding of [^{3}H]-angiotensin II to intact cells as well as to angiotensin II induced inositol phosphate accumulation is measured.

Results. Both antagonists inhibit specific [^{3}H]-angiotensin II binding to AT_1 receptors in these cells, with IC_{50} values of 5.9 and 5.2 nM, respectively. Preincubation of the cells with LY301875 results in a decline of up to 80 % of the maximal angiotensin II-stimulated inositol phosphate (IP) production. A near complete decline of the maximal response is observed for LY303336. This insurmountable inhibition is attenuated for both antagonists when losartan is included during the preincubation of the cells.

Conclusions. Functional recovery experiments, in which antagonist-preincubated cells are washed and exposed to fresh media, suggest that the insurmountable inhibition by LY301875 and LY303336 is related to their relatively slow dissociation from the AT_1 receptors. As already described for losartan and the derived insurmountable AT_1 antagonists candesartan, EXP3174, and irbesartan, coincubation experiments reveal that LY301875 and LY303336 interact with the AT_1 receptor in a manner that is competitive with angiotensin II.

KEY WORDS: LY301875; LY303336; CHO cells; AT_1 receptor; binding.

INTRODUCTION

There is considerable interest in the therapeutic use of selective antagonists for the angiotensin II type 1 (AT_1) receptor in the treatment of hypertension and congestive heart failure (1). Initial attempts to develop therapeutic active AT_1 blocking agents failed due to antagonists that are peptides that had very limited or no oral activity. Moreover, the peptide AT_1 antagonist saralasin behaved as a partial agonist (2). A major breaktrough was achieved by the discovery of the selective and nonpeptide AT_1 antagonist losartan (3). Several derivatives based on the biphenyltetrazole or N-benzylimidazole substructure of losartan were subsequently identified as very potent AT_1 receptor antagonists (4). They have traditionally been tested for their ability to inhibit angiotensin II-induced contraction of rabbit aortic rings/strips, a system with very small receptor reserve (5). In these experiments, the tissue is pre-equilibrated with the antagonist before the consecutive addition of increasing concentrations of angiotensin II. Based on their different capabilities to affect the concentration–contractile response curve of angiotensin II, the antagonists have been divided in two categories (6–11). Surmountable antagonists such as losartan, tasosartan, and eprosartan produce a concentration-dependent rightward shift of the curve without affecting the maximal response. Insurmountable antagonists, in addition, depress the maximal contractile response to angiotensin II. This depression may be almost complete (e.g., for candesartan) or only partial (e.g. for irbesartan, valsartan and EXP3174). Recently it was demonstrated in Chinese Hamster Ovary (CHO) cells expressing human AT_1 receptors that these antagonists inhibit the angiotensin II-mediated inositol phosphate (IP) production in a competitive manner and, hence, that they bind to a common or overlapping binding site on the receptor (12). To deal with the often partial insurmountable effects of many AT_1 receptor antagonists, it was proposed that the antagonist–receptor complex can adopt a fast reversible/surmountable and a slow reversible/insurmountable state (13). The proportion of both states that are dependent on the nature of the antagonist, determines the extent of its insurmountable inhibition.

Since these recent insights were gained only by studying the inhibitory properties of antagonists with the common biphenyltetrazole moiety of losartan; i.e. candesartan, EXP3174, irbesartan, and losartan themselves, it is not clear whether they can be applied to dissimilar antagonists. In this context, a number of molecules which do not possess the biphenyltetrazole moiety have been shown to behave as potent AT_1 receptor antagonists as well (4,14,15). Among these, the polysubstituted 4-aminoimidazole derivatives LY301875 and LY303336 possess three ionizable groups at physiological pH and three chiral centers. These compounds produce an antihypertensive activity after oral application and, when preincubated with isolated rabbit aorta, they produce an insurmountable inhibition of angiotensin II-induced contraction (14,15). Based on their structure (Fig. 1) it was also suggested that they bind to an additional subsite of the AT_1 receptor not accessed by losartan. These differences prompted us to investigate the inhibitory properties of LY301875 and LY303336 on CHO cells expressing human recombinant AT_1 receptors. As for the biphenyltetrazole derivatives, LY301875 and LY303336 antagonize the angiotensin II-mediated IP production in a competitive fashion, and their insurmountable effect in preincubation experiments can be linked to their slow dissociation from the AT_1 receptors.

MATERIALS AND METHODS

Materials

LY301875 (cis-4-[-(carboxymethyl)phenoxy]-1-[1-oxo-2(**S**)-[4-[(2-sulfobenzoyl)amino]-1H-imidazol-1-y1]octyl]-L-

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ABBREVIATIONS: AT_1, Angiotensin II type 1; CHO, Chinese Hamster Ovary cells; DMEM Dulbecco's Minimal Essential Medium; IP Inositol phosphates.

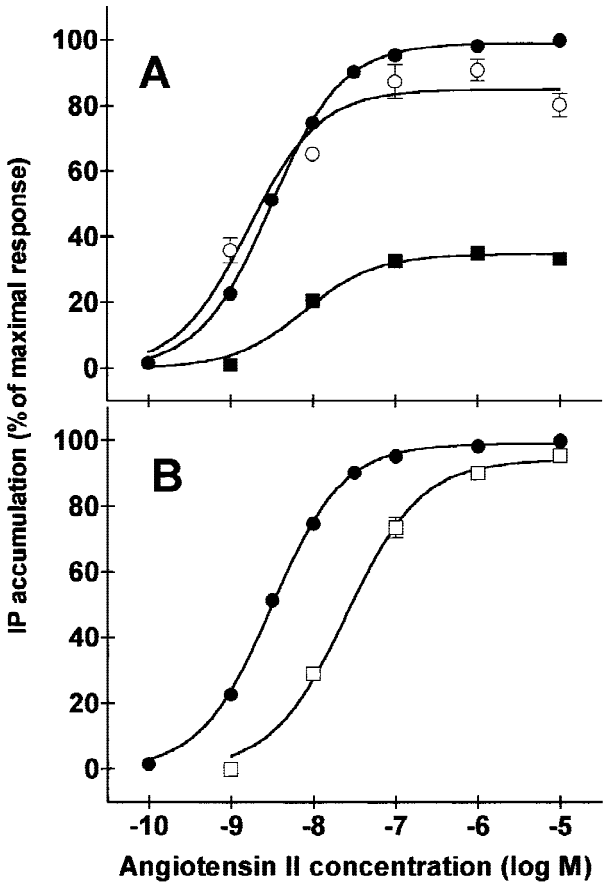

Fig. 1. Effect of LY301875 on concentration–response curves of angiotensin II-induced IP production in CHO-AT_1 cells. A. Effect of 10 (○) or 100 nM (■) LY301875 pre-incubation. B. Effect of simultaneous application of 1 μM LY301875 (□). In the control curves (●), the cells were not pretreated with the antagonist. The corresponding pEC_{50} and E_{max} values are given in Table 1, and the values are given as percentage of the E_{max} without antagonist pre-treatment.

proline) and LY303336 (cis-4-[-(carboxymethyl)phenoxy]-1-[1-oxo-2(**R**)-[4-[(2-sulfobenzoyl)amino]-1H-imidazol-1-y1]-octyl]-L-proline) were kindly provided by the Lilly Research Laboratories (Indianapolis, USA). Candesartan, losartan, EXP3174, and irbesartan were obtained from AstraZeneca (Mölndal, Sweden), and angiotensin II was obtained from Sigma. All other chemicals were of the highest grade commercially available.

Cloning of the Human AT_1 Receptor and Expression in CHO Cells

The cloning procedure has been described previously (16). Briefly, the cDNA for the human angiotensin II AT_1 receptor was isolated and multiplied using primers corresponding to the 5′ (ATGATTCTCAACTCTTCTACT) and 3′ (TCACTCAACCTCAAAACATGG) ends of the human AT_1 receptor sequence (17) from cDNA of the human adrenal gland (Clontech, U.S.A.). A polymerase chain reaction (PCR) reaction mixture containing 1 ng cDNA was incubated at 93°C for 45 s, 60°C for 20 s, and 72°C for 60 s for a total of 30 cycles. The generated coding region of human AT_1 receptor was cloned in a PCR3 vector (InVitrogen) and sequenced using the sequenase kit (Amersham, U.K.). It was subsequently transfected in CHO-K1 cells using Lipofectin (Life-Technologies, U.S.A.) according to the manufacturer's instructions. After 72 hours of transfection, cells were harvested and replated in Dulbecco's Minimal Essential Medium (DMEM) containing 1 mg/ml geneticin. Individual clones were collected, grown, and analyzed for binding to [^{125}I]-angiotensin II. The clone denoted as CHO-AT_1 cells with maximum binding capacity was further used in this study and was cultured in 75 cm^2 flasks in DMEM, which was supplemented with L-glutamine (2 mM), 2% of a stock solution containing 5000 I.U./ml penicillin and 5000 μg/ml streptomycin (Life Technologies), 1% (v/v) of a solution of Minimal Essential Medium (MEM) containing nonessential amino acids, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (Life Technologies). The cells were grown in 24 well plates for [^{3}H]-angiotensin II binding as well as angiotensin II-induced IP accumulation.

[^{3}H]-Angiotensin II Binding

Before the experiment, the cells were washed twice with 0.5 ml per well of DMEM at room temperature. After removal of the medium, 500 μl DMEM was added and the plate was then left for 15 min at 37°C. Competition binding experiments were performed by preincubation of the cells with increasing concentrations of unlabelled antagonists for 30 min at 37°C and further incubation for 30 min at 37°C with 1 nM [^{3}H]-angiotensin II. At the end of each incubation, the cells were briefly washed twice with (phosphate buffered saline) PBS containing 0.132 g/L $CaCl_2 2H_2O$, 0.2 g/L KCl, 0.2 g/L KH_2PO_4 0.1 g/L $MgCl_2.6H_2O$, 8 g/L NaCl, and 1.44 g/L $Na_2HPO_4.2H_2O$) at 4°C. Subsequently, the cell-bound radioactivity in each well was solubilized with 500 μl sodium hydroxide (0.2 M) and counted for 3 min in a liquid scintillation counter after adding 3 ml scintillation liquid (Optisafe of Wallac). Nonspecific binding, measured in the presence of 0.1 μM unlabelled candesartan, was subtracted from the total binding to yield specific [^{3}H]-angiotensin II binding.

IP Accumulation

The cells were plated in 24 well plates and cultured to near confluence. The medium was replaced by supplemented DMEM (see Cell Culture section) containing 1 μCi/ml myo[^{3}H]-inositol, and the cells were further grown for 20 h in 5% CO_2 at 37°C, until confluence. To investigate the effect of LY301875 and LY303336 on angiotensin II concentration–response curves, the cells were first washed twice with DMEM and left in 400 μl of DMEM containing 10 mM LiCl for 15 min at 37°C. Preincubations were initiated by addition of 50 μl medium without (controls) or with LY301875 or LY303336 and proceeded at 37°C for 30 min after which the cells were further incubated with angiotensin II or medium alone (for basal IP accumulation) at 37°C for 5 min. Coincubations were performed by simultaneous addition of antagonist and angiotensin II to the cells and lasted for 5 min at 37°C.

For the functional recovery experiments, the cells were first washed twice with DMEM (500 μl per well). After antagonist preincubation at 37°C for 30 min, the cells were washed three times with 500 μL DMEM, left in DMEM either with or without 1 μM losartan for the indicated periods

of time (i.e., washout time) at 37°C and washed three times with DMEM again. Subsequently, they were incubated with 0.1 μM angiotensin II or medium alone (for basal IP accumulation) at 37°C for 5 min. The IP accumulation represented the measurement of mono-, bis-, and trisphosphates as described by Vanderheyden *et al.* (16). For the concentration–effect curves the responses were given as a percentage of the maximal angiotensin II response in the absence of antagonist pre-treatment. For the recovery experiments, responses were given as a percentage of the matching control agonist stimulation without antagonist pre-treatment.

RESULTS

Effect of LY301875 and LY303336 on the Binding of [^{3}H]-Angiotensin II

Preincubation of the CHO-AT_1 cells for 30 min with LY301875 and LY303336 (Table I) caused a concentration-dependent inhibition of the specific binding of 1 nM [^{3}H]-angiotensin II. Maximal inhibition occured to the same extent as with angiotensin II and the antagonists candesartan, EXP3174, irbesartan, and losartan. The estimated Hill coefficients of the curves were similar, indicating the presence of a single population of binding sites (Table I). The potency order was candesartan ≅ angiotensin II EXP3174 > LY303336 ≅ irbesartan > LY301875 > losartan.

Angiotensin II-Induced IP Production

Incubation of the CHO-AT_1 cells with angiotensin II for 5 min produced a concentration-dependent increase of IP accumulation with an EC_{50} of 3.1 nM. Preincubation of the cells for 30 min with 1, 10, and 100 nM of LY301875 and LY303336 resulted in a concentration-dependent rightward shift of the concentration–response curve of angiotensin II along with a decline of the maximal response (Figs. 1A and 2A and Table 1). Whereas this decline was near to completion with 100 nM LY303336, only a partial reduction of the maximal response was seen after preincubation with 100 nM LY301875. As depicted in Table II this insurmountable inhibition by LY301875 and LY303336 was attenuated when 1 μM losartan was included throughout the experiment.

In contrast to the above preincubation experiments, simultaneous incubation of the cells with angiotensin II and 1 μM LY301875 or LY303336 resulted in a rightward shift of the concentration–response curves without a noticable change of the the maximal response (Figs. 1B and 2B). An alternative way to represent antagonistic action eq. is to measure the angiotensin II (0.1 and 10 μM)-induced IP production after pretreatment of the cells with increasing concentrations of LY301875 and LY303336 (between 0.1 nM and 10 μM). As shown in Fig. 3, the obtained antagonist concentration–inhibition curves were biphasic for LY301875. The most potent component represents 80% of the inhibition. It is insensitive to the angiotensin II concentration, and can be referred to as insurmountable inhibition. The less potent component depends on the angiotensin II concentration and represents surmountable inhibition. In line with the angiotensin II concentration–response curve, the inhibition by LY303336 was found to be approximately 95% insurmountable.

The reversal of the antagonist inhibition was assessed by washout experiments involving preincubation of the CHO-AT_1 cells with 100 nM LY301875 or LY303336, washing and exposure to fresh medium for the indicated periods of time before measuring the maximal angiotensin II-mediated IP production (i.e., 5 min incubation with 0.1 μM angiotensin II). As shown in Fig. 5, the maximal response recovered very slowly in LY303336-pretreated cells and slightly faster in LY301875-pretreated cells. The same experiments were also carried out with washout medium containing 1 μM losartan. At this concentration, losartan effectively prevents the potential re-association of initially added antagonists to the receptor without affecting the maximal angiotensin II-induced IP production (12). As shown in Fig. 4, losartan did not noticeably affect the rate of recovery of the maximal response in LY301875- and LY303336-pretreated cells.

Table 1. [^{3}H]-Angiotensin II Competition Binding Parameters to CHO-AT_1 Cells

Compound	–log IC_{50}^a	Hill coefficient
Candesartan	9.00 ± 0.079	1.29 ± 0.12
Angiotensin II	8.95 ± 0.014	1.05 ± 0.02
EXP3174	8.91 ± 0.027	0.93 ± 0.04
Irbesartan	8.23 ± 0.120	0.70 ± 0.04
LY303336	8.27 ± 0.065	1.02 ± 0.08
LY301875	8.01 ± 0.056	1.16 ± 0.06
Losartan	7.65 ± 0.042	1.25 ± 0.19

[a] IC_{50} values are determined by nonlinear regression analysis of competition curves.

Table 2. Inhibition of Angiotensin II-Induced IP Accumulation

Condition	–log EC_{50}	E_{max} (% of control)
Control	8.51 ± 0.02	100 ± 2
1 nM preincubation LY303336	8.60 ± 0.10	92 ± 2
10 nM preincubation LY303336	8.42 ± 0.13	50 ± 2*
100 nM preincubation LY303336	7.47 ± 0.08	7 ± 2*
100 nM preincubation LY303336 + 1 μM losartan	6.80 ± 0.08	62 ± 2*
100 nM coincubation LY303336	8.10 ± 0.05	98 ± 2
1000 nM coincubation LY303336	7.46 ± 0.17	89 ± 6
1 nM preincubation LY301875	8.91 ± 0.09	103 ± 4
10 nM preincubation LY301875	8.78 ± 0.15	85 ± 4*
100 nM preincubation LY301875	8.11 ± 0.10	35 ± 1*
100 nM preincubation LY301875 + 1 μM losartan	6.81 ± 0.08	68 ± 3*
100 nM coincubation LY301875	8.70 ± 0.13	103 ± 4
1000 nM coincubation LY301875	7.60 ± 0.08	94 ± 3

Note: The EC_{50} and E_{max} values are determined by nonlinear regression analysis of angiotensin II concentration–response curve.
* statistically significant different ($p < 0.05$) from control values as determined by unpaired t-tests.

DISCUSSION

Most of the reported AT_1 antagonists are based on the biphenyltetrazole substructure of losartan or on the N-benzylimidazole series from which losartan was originally derived (4). The inhibitory properties of four of such compounds, i.e., candesartan, EXP3174, irbesartan, and losartan were recently described in CHO cells expressing human re-

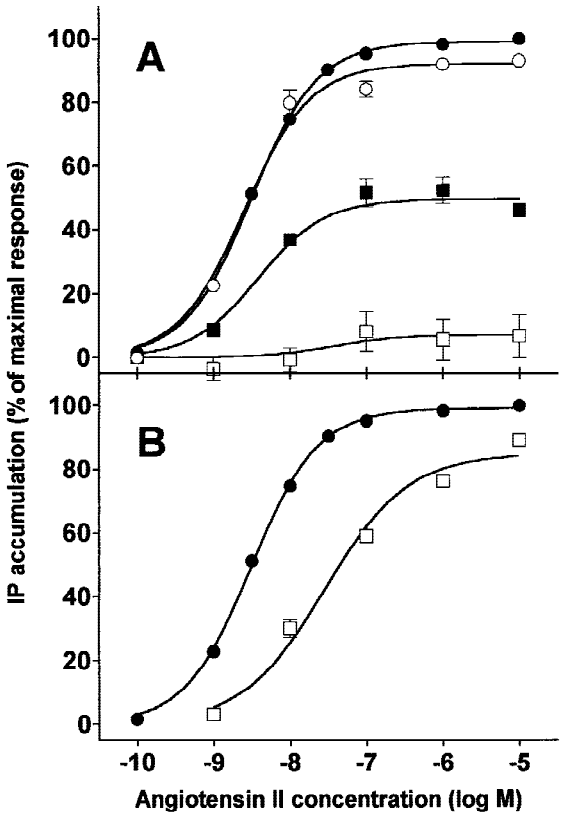

Fig. 2. Effect of LY303336 on concentration–response curves of angiotensin II-induced IP production in CHO-AT_1 cells. A. Effect of 1 (○), 10 (■) or 100 nM (□) LY303336 pre-incubation. B. Effect of simultaneous application of 1 μM LY303336 (□). In the control curves (●), the cells were not pretreated with the antagonist. The corresponding pEC_{50} and E_{max} values are given in Table 1, and the values are given as percentage of the E_{max} without antagonist pretreatment.

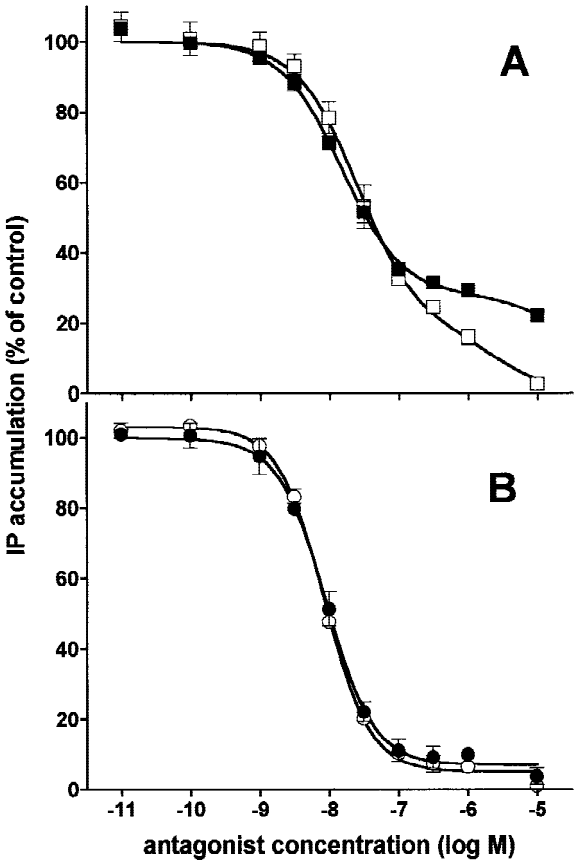

Fig. 3. Antagonist concentration–inhibition curves of LY301875 (panel A) and of LY303336 (panel B). CHO-AT_1 cells were preincubated for 30 min at 37°C with the antagonist, after which the cells are further incubated for 5 min with 10 μM (■,●) or 0.1 μM (□,○) angiotensin II. The resulting IP accumulation is given as a percentage of the control response in the absence of antagonist.

combinant AT_1 receptors (16). It was found in co-incubation experiments that they act competitively with angiotensin II and that they bind in a manner mutually exclusive to the receptor (12,13). Furthermore, the extent of insurmountable inhibition they produced in preincubation experiments was found to be related to their rate of dissociation from the receptor (18). In these studies, evidence was presented that such antagonist–receptor complexes can adopt a fast and a slow reversible state, and that the equilibrium between both states, which determines the extent of insurmountable inhibition, is dependent on the nature of the antagonists (13).

To find out whether such mechanisms are also relevant for nonpeptide antagonists whose basic structure is different from that of losartan, we have investigated the inhibitory properties of LY301875 and LY303336. These two AT_1 receptor antagonists are representatives of a family of polysubstituted 4-aminoimidazole compounds (Fig. 5). In this vein, it has been proposed that antagonists such as losartan are able to fill several subsites of the receptor that are are important for the recognition of angiotensin II—They include a lipophilic pocket that accommodates an alkyl chain and a basic amino acid residue that interacts electrostatically with an acidic group of the ligand (19,20). However, a comparison of the three-dimensional structures of losartan and LY303336 indicated that the latter antagonist may reach an additional, previously unknown subsite of the AT_1 receptor (15). In this respect, the insurmountable inhibition by antagonists such as candesartan and EXP3174 could be ascribed to the presence and correct positioning of two negatively-charged groups (a carboxyl and a tetrazole moiety) when compared to the surmountable inhibition by losartan which has only the tetrazole group. Further modeling and receptor mutagenesis work is necessary to elucidate whether this notion is also valid for LY301875 and LY303336 that possess three acidic groups.

Although their structure and postulated mode of interaction with the receptor is different from that of losartan and its analogues, it was found that LY301875 and LY303336 interact with the human recombinant AT_1 receptors in nearly the same way as previously reported for the insurmountable losartan-related antagonists candesartan and EXP3174. First, LY301875 and LY303336 are competitive with angiotensin II as well as with losartan. To find out whether the inhibitory effect of LY301875 and LY303336 is competitive with angiotensin II, it is mandatory that both the antagonist and the agonist are applied simultaneously to the receptor (13). As illustrated in Figs. 1 and 2, co-incubation of angiotensin II with LY301875 or LY303336 resulted in a parallel rightward shift of the concentration–response (IP accumulation) curves without affecting the maximal response. In combination with the absence of receptor reserve in the CHO-AT_1 cells (5),

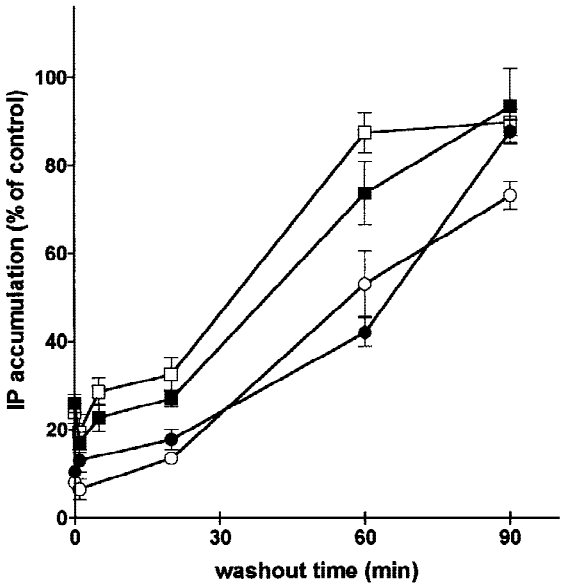

Fig. 4. Recovery of angiotensin II (0.1 μM, 5 min)-induced IP production in CHO-AT_1 cells after preincubation with 100 nM LY301875 (■,□) or 100 nM LY303336 (●,○) and wash-out of the cells with fresh medium (filled symbols) or medium containing 1 μM losartan (open symbols). Data are expressed as a percentage of the matching control agonist stimulation without antagonist pretreatment.

these findings therefore clearly establish that LY301875 and LY303336 inhibit the angiotensin II responses in a competitive fashion. Moreover, the ability of losartan to alleviate the insurmountable inhibition by LY301875 and LY303336 in preincubation experiments (please see below) implies that both types of antagonists bind to a common or overlapping binding site on the AT_1 receptor. The same behavior has been reported for biphenyltetrazole or N-benzylimidazole antagonists candesartan, EXP3174, and irbesartan (12).

Second, the insurmountable effect of LY301875 and LY303336 can be ascribed to their long-lasting occupancy of the receptors. When CHO-AT_1 cells are preincubated with these compounds, there is a pronounced insurmountable inhibition of angiotensin II-induced IP production. Several theories have been proposed to explain this particular behavior of AT_1 receptor antagonists. These theories include the presence of allosteric binding sites on the receptor (21), slowly interconverting receptor conformations (22,23), slow removal of the antagonist from the receptor from tissue compartments (24,25) or from cells or matrices surrounding the receptor (26), coexistence of different receptor subpopulations (21), and even the ability of antagonists to modulate the amount of internalized receptors (8). However, there is now increasing evidence that insurmountable inhibition of losartan-like antagonists is caused by their relatively slow dissociation rate from the receptor (27–29). In this context, the recovery of angiotensin II-induced IP production in candesartan-preincubated CHO-AT_1 cells was shown to be very slow and to coincide with the dissociation rate of [^{3}H]-candesartan binding to the AT_1 receptor (18). As a result of their long half-life, the candesartan–receptor complexes undergo almost no dissociation during the ensuing, relatively short exposure of the cells to angiotensin II, so that almost no stimulation can take place even at very high angiotensin II concentrations. A similar explanation may be advanced for LY301875 and LY303336. Indeed, when pre-exposed to the receptor, the inhibitory action of these antagonists is long-lasting in washout experiments: The half maximal recovery of angiotensin II-induced IP production ocurred at approximately 60 min for LY303336 and approximately 40 min for LY301875.

Third, LY301875- and LY303336-receptor complexes may adopt two states. Whereas the partial nature of the insurmountable inhibition by both antagonists is obvious by analyzing angiotensin II concentration–response curves, it can also be delineated by antagonist concentration–inhibition curves. In this approach, the effect of a single, high concentration of angiotensin II is measured after preincubation with a wide concentration range of each antagonist. Nonlinear regression analysis of the resulting biphasic inhibition curves allows an accurate quantification of the proportion of insurmountable inhibition, i.e., 80% for LY301875 and up to almost 95% for LY303336. As for the losartan-like insurmountable antagonists (13), these percentages no longer varied when the preincubation time with LY303336 or LY301875 was prolonged up to 120 min (data not shown). These data are

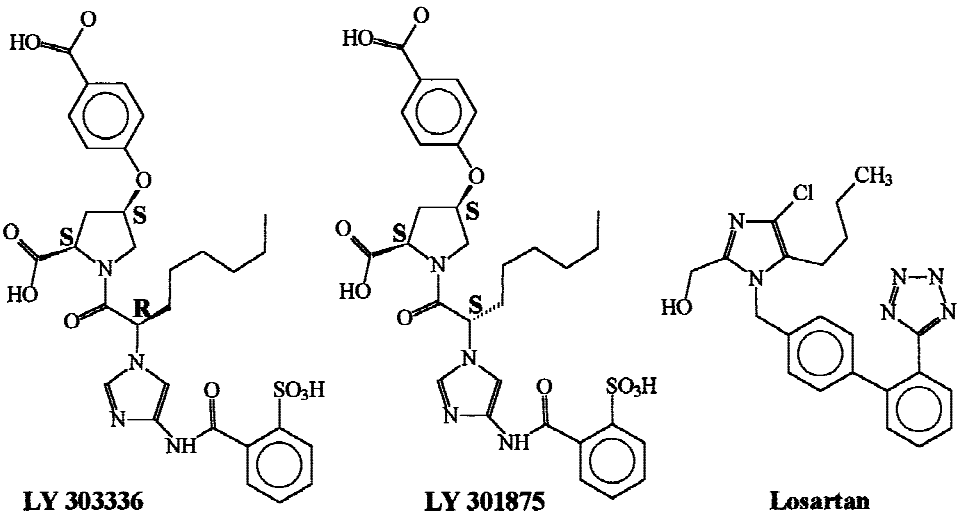

Fig. 5. Chemical structures of LY303336, LY301875, and losartan.

compatible with the ability of LY303336- and LY301875-receptor complexes to adopt an insurmountable/long lasting and a surmountable/fast reversible state, and that there is an equilibrium between both states which is dependent on the antagonist structure. Several avenues will need to be explored to understand the molecular significance of the fast reversible and tight binding states of the antagonist–AT_1 receptor complexes. Among the many theoretical possibilities, the distinction between both states could reside at the level of the receptor conformation, its association with other proteins, or even its subcellular localization.

Despite a similar degree of insurmountable antagonism, LY303336 and LY301875 differ from the losartan-related antagonists candesartan and EXP3174 by their lower overall affinity at the AT_1 receptor and by their lack of rebinding to the receptor in wash-out experiments. LY303336 or LY301875 were found to inhibit specific [^{3}H]-angiotensin II binding to intact CHO-AT_1 cells with nanomolar IC_{50} values. These are above those for candesartan and EXP3174, but in the same range as the IC_{50} value of the weakly insurmountable antagonist irbesartan. Previous wash-out experiments with [^{3}H]-candesartan on CHO-AT_1 cells revealed that, because of its high affinity for the AT_1 receptor, dissociated [^{3}H]-candesartan molecules may bind again to the receptor, resulting in an apparent slower dissociation rate (18). Experimentally, this "rebinding" phenomenon (18,30) could be prevented by inclusion of an excess of unlabeled receptor ligand such as losartan. Rebinding of candesartan as well as EXP3174 could also be demonstrated by a faster recovery of the angiotensin II-mediated IP response in wash-out experiments when losartan was included in the wash-out medium (12,18). In contrast, no rebinding appears to occur for LY301875 and LY303336, as losartan does not noticeably affect the recovery of angiotensin II-mediated response from both antagonists, likely due to their relatively lower affinity for the receptor.

In conclusion, despite major structural differences between LY301875 and LY303336 and losartan-related insurmountable AT_1 receptor antagonists, the present data suggest that they may bind to overlapping sites of the receptor. As for these other antagonists, the insurmountable behavior of LY301875 and LY303336 in pre-incubation experiments can be attributed to their slow dissociation of the receptor, and the incomplete nature of their insurmountable effect is compatible with their ability to form insurmountable/long lasting and insurmountable/ fast reversible complexes with the receptor. However, LY301875 and LY303336 are distinct from the equally insurmountable antagonists EXP3174 and candesartan by their relatively lower affinity for the receptor and the resulting lack of rebinding in wash-out experiments.

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