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In vitro pharmacology of an angiotensin AT_1 receptor antagonist with balanced affinity for AT_2 receptors

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Abstract

L-163,017 (6-[benzoylamino]-7-methyl-2-propyl-3-[[2′-(*N*-(3-methyl-1-butoxy) carbonylaminosulfonyl)[1,1′]-biphenyl-4-yl]methyl]-3*H*-imidazo[4,5-*b*]pyridine) inhibited specific ^{125}I-[Sar^1,Ile^8]angiotensin II binding to angiotensin AT_1 receptor (K_i = 0.11–0.20 nM) in rabbit aorta, rat adrenal and human angiotensin AT_1 receptor in CHO (Chinese hamster ovary transformed) cells and to AT_2 receptor (K_i = 0.14–0.23 nM) in rat adrenal and brain receptors. L-163,017 also had a high affinity in the presence of bovine serum albumin (2 mg/ml), for angiotensin AT_1 and AT_2 receptors on human adrenal (K_i 3.9 and 4.3 nM), aorta (K_i 0.45 and 0.96 nM) and kidney (K_i 3.6 and 2.3 nM). The much higher K_i values in human tissues were likely due to the presence of bovine serum albumin in the binding assay buffer since L-163,017 had K_i values of 0.13 ± 0.04 and 2.0 ± 0.04 nM in the absence and presence of bovine serum albumin, respectively, in inhibiting ^{125}I-[Sar^1,Ile^8]angiotensin II binding to angiotensin AT_1 receptor in rat adrenal membranes. Scatchard analysis of ^{125}I-[Sar^1,Ile^8]angiotensin II binding in the presence of bovine serum albumin (2 mg/ml) in rabbit aorta and bovine cerebellum indicated a competitive interaction of L-163,017 with angiotensin AT_1 and AT_2 receptors (K_i values 2.5 and 2.1 nM respectively). L-163,017 inhibited angiotensin II-induced aldosterone release in rat adrenal demonstrating that L-163,017 acted as a competitive antagonist (pA_2 = 9.9) and lacked agonist activity. L-163,017 also inhibited angiotensin II responses in rat vascular tissues. The specificity of L-163,017 was shown by its lack of activity on the above functional responses produced by other agonists and in several binding assays.

Keywords: Angiotensin II receptor subtype; Hypertension; L-163,017; Renin-angiotensin system

1. Introduction

The role of the renin-angiotensin system in the regulation of blood pressure, electrolyte and fluid balance in normal physiological and various pathophysiological states is well supported by the clinical uses of angiotensin converting enzyme inhibitors (Ondetti et al., 1977; Cody, 1986; Waeber, 1990). The potential therapeutic advantages of angiotensin II receptor antagonists compared to angiotensin converting enzyme inhibitors have been disucssed (Smith et al., 1992). The discovery of losartan (Dup-753/MK-954) as a selective nonpeptide angiotensin II receptor antagonists (Chiu et al., 1990; Wong et al., 1990a) was followed by many newer antagonists (Mantlo et al., 1991; Chang et al., 1992, 1994; Siegl et al., 1992; Edwards et al., 1991; Shibouta et al., 1992; Criscione et al., 1993; Cazaubon et al., 1993; Oldham et al., 1992; Kivlighn et al., 1995a; Ashton et al., 1993a,b).

The existence of angiotensin II receptor subtypes, namely angiotensin AT_1 and AT_2 subtypes in various tissues was supported by selective angiotensin AT_1 and AT_2 antagonists (Chang and Lotti, 1989, 1990; Whitebread et al., 1989; Chiu et al., 1989; Bumpus et al., 1991). The angiotensin II receptor subtype having a

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high affinity for losartan has been designated angiotensin AT_1 receptor and the receptor subtype having a high affinity for PD123177 (1-(3-methyl-4-aminophenyl) methyl-5- diphenylacetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid) as angiotensin AT_2 receptor (Bumpus et al., 1991). The angiotensin AT_1 receptors are associated with the classical functions of angiotensin II in producing vasoconstriction and aldosterone secretion while the function of the angiotensin AT_2 receptor is less clear (Smith et al., 1992). Losartan is currently in advanced clinical studies (Nelson et al., 1991). However, recent reports have suggested the possible functional roles of angiotensin AT_2 receptor in growth and development (Grady et al., 1991; Tsutsumi and Saavedra, 1991), proliferation of neointima following balloon angioplasty (Janiak et al., 1992) and collagen synthesis (Brilla, 1992).

It is unclear whether a balanced (i.e. equipotent at both angiotensin AT_1 and AT_2 sites) angiotensin receptor antagonist will have advantages over an angiotensin AT_1-selective angiotensin receptor antagonist. However, it is now well established in patients receiving angiotensin AT_1-selective angiotensin receptor antagonists, such as losartan, that circulating plasma levels of angiotensin II are significantly elevated (Goldberg et al., 1993). Therefore it is conceivable that in the presence of angiotensin AT_1 receptor blockade the increased circulating levels of angiotensin II could interact with the angiotensin AT_2 receptor subtype to produce an undesirable biological response. Therefore, a balanced angiotensin AT_1/AT_2 receptor antagonist may be therapeutically advantageous over a selective angiotensin AT_1 antagonist. All earlier reported nonpeptide angiotensin II receptor antagonists have selectivity for either angiotensin AT_1 or AT_2 receptor with the exception of the recent reports which described compounds having similar affinity for angiotensin AT_1 and AT_2 receptors (De Laszlo et al., 1993; Glinka et al., 1994; Mantlo et al., 1994). In this report, we describe the in vitro pharmacology of the most interesting compound identified from these chemical series – L-163,017 (6-[benzoylamino]-7-methyl-2-propyl-3-[[2′-(*N*-(3-methyl-1-butoxy) carbonylaminosulfonyl)[1,1′′]-biphenyl-4-yl]methyl]-3*H*-imidazo[4,5-*b*]-pyridine). This compound has the desired in vivo pharmacological properties, namely it has good oral bioavailability and long duration as described in our accompanying paper (Kivlighn et al., 1995b).

2. Materials and methods

2.1. Angiotensin II radioligand receptor binding assays

The preparation of membranes from various tissues and specific ^{125}I-[Sar^1,Ile^8]angiotensin II binding assays were as described in our previously published methods (Chang and Lotti, 1991; Chang et al., 1992; Chen et al., 1992) with the exception that bovine serum albumin was omitted in the binding assay buffers for most assays. Bovine serum albumin (2 mg/ml) was added to the binding assay buffer for human tissues and for rabbit aorta and bovine cerebellum when Scatchard analysis was performed because bovine serum albumin improves the ratio of specific binding to nonspecific binding. Cloned human angiotensin AT_1 receptor membranes were prepared from confluent CHO (Chinese hamster ovary transformed) cells expressing the human angiotensin AT_1 receptor gene (gift of Dr. Pamela Benfield of DuPont-Merck) by scraping the cells off the flask into 5–10 ml of phosphate-buffered saline with a cell scraper. The cells were centrifuged for 10 min at 1200 rpm and then homogenized in 50 mM Tris[hydroxymethyl]aminomethane hydrochloride (pH 7.7) with a glass homogenizer. The homogenate was centrifuged at $40\,000 \times g$ for 10 min. The membrane pellets were resuspended in approximately 10 ml for each 162 cm^2 flask of cells in binding assay buffer (50 mM Tris[hydroxymethyl]aminomethane hydrochloride pH 7.4, 5 mM magnesium chloride, 0.2 mg/ml bacitracin, 0.2 mg/ml soybean trypsin inhibitor and 0.1 mM *o*-phenanthroline). For ^{125}I-[Sar^1,Ile^8]angiotensin II binding assays, the membrane pellets were resuspended in appropriate volumes of binding assay buffers. The tissue concentrations in mg original wet weight per ml given in parentheses were: rabbit aorta (33), rat adrenal (0.5–1.0), rat brain (6.3), human adrenal (10), aorta (33), kidney (5) and uterus (20). For adrenal, brain, kidney and uterus tissues, the binding assay buffer contained 10 mM sodium phosphate, 5 mM disodium ethylenediamine tetracetate (pH 7.4), 120 mM sodium chloride, 0.1 mM phenylmethane sulfonyl fluoride, 0.2 mg/ml soybean trypsin inhibitor, 0.02 mg/ml *o*-phenanthroline and 0.14 mg/ml bacitracin. Bovine cerebellum membranes (DuPont New England Nuclear) were diluted 6-fold in the same binding assay buffer for rat brain. The binding assay buffer for aorta consisted of 50 mM Tris[hydroxymethyl]aminomethane hydrochloride, 5 mM magnesium chloride, 0.2 mg/ml bacitracin and soybean trypsin inhibitor (0.2 mg/ml). In some studies using rat adrenal, human serum albumin (50 mg/ml) was added to assess the effect of human serum albumin on the IC_{50} values in the ^{125}I-[Sar^{1}Ile^8]angiotensin II binding assay.

For ^{125}I-[Sar^1,Ile^8]angiotensin II binding assays, 10 μl of buffer (for total binding), or [Sar^{1}Ile^8]angiotensin II (1 μM) (for nonspecific binding) or test compounds (for displacement) and 10 μl ^{125}I-[Sar^1,Ile^8]angiotensin II (23–58 pM, unless otherwise specified) were added to duplicate or triplicate tubes. Receptor membranes (250 or 500 μl) were added to each tube to initiate the binding reaction. The final volumes were 270 μl for the

aorta and 520 μl for other tissues. The reaction mixtures were incubated at 37°C for 90 min. The reaction was terminated by filtration under reduced pressure through glass-fiber GF/B filters and washed immediately 4 times with 4 ml of ice-cold Tris[hydroxymethyl]aminomethane hydrochloride (5 mM; pH 7.4) containing 0.15 M sodium chloride. The radioactivity trapped on the filters was counted using a gamma counter.

In all tissues specific ^{125}I-[Sar^1,Ile^8]angiotensin II binding was defined as the difference between total and nonspecific binding. IC_{50} values were determined by regression analysis of displacement curves. In tissues in which both angiotensin AT_1 and AT_2 receptor subtypes were present, the IC_{50} values on angiotensin AT_1 and AT_2 receptors were determined in the presence of 1 μM of PD121981 (WL-19, 1-(3-methyl-4-methoxyphenyl)methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid) or losartan to prevent the radioligand binding to angiotensin AT_2 and AT_1 receptors respectively (Chang and Lotti, 1990,1991). K_i values were calculated according to the formula $K_i = IC_{50}/(1 + [L]/K_d)$ (Cheng and Prusoff, 1973).

2.2. Other radioligand binding assays

These studies were conducted under contract with Panlabs for a variety of receptor binding assays including adenosine A_1, A_2, bradykinin B_2, cholecystokinin A, B, galanin, histamine H_3, insulin, interleukin-1α, kainate, leukotriene B_4, muscarinic M_1, M_2, neurokinin NK_1, neuropeptide Y, *N*-methyl-D-aspartic acid (NMDA), platelet activating factor, 5-hydroxytryptamine (5-HT) (5-HT_{1A}, 5-HT_{1B}), sodium channel, thyrotropin releasing hormone and vasoactive intestinal polypeptide.

2.3. Angiotensin II-induced aldosterone release in rat dispersed adrenal capsular cells

The method for the preparation of rat adrenal dispersed capsular cells was according to published procedures (Douglas et al., 1978). The cell suspensions (0.5 ml in Medium 199 containing 2 mg/ml bovine serum albumin, 0.14 mg/ml bacitracin, 0.2 mg/ml soybean trypsin inhibitor, 50 units/ml penicillin and 50 μg/ml streptomycin) were added to triplicate tubes containing 10 μl buffer (basal release) or angiotensin II (10^{-11}–10^{-7} M) with or without L-163,017 (20 nM). The cells were incubated at 37°C for 2 h under 95% oxygen and 5% carbon dioxide atmosphere and then centrifuged at $1000 \times g$ for 15 min. Supernatants were carefully removed and aliquots (100 μl) were used in a radioimmunoassay for aldosterone using a kit from Radioassay System Laboratories (Carson, CA, USA).

2.4. Isolated rat pulmonary artery

Male, Charles River Sprague-Dawley rats (300–400 g) were killed by cervical dislocation. The main pulmonary artery was removed and placed in a Petri dish containing Krebs salt solution (37°C) of the following composition: sodium chloride, 118 mM; potassium chloride, 4.7 mM; calcium chloride, 2.5 mM; potassium phosphate (monobasic), 1.2 mM; magnesium sulfate, 1.2 mM; sodium bicarbonate, 25 mM; dextrose, 11 mM. Excess lipid material was cleaned from the tissue with the aid of a dissecting microscope. A ring (~ 4–5 mm in length) was mounted on a thin (34 gauge) stainless steel wire bent into a triangular shape (length of sides ~ 5 mm). The apex of the triangle (farthest from the ring) was connected to a glass tissue holder with 4-0 surgical silk. The tissue was mounted on another triangle and its apex was connected via surgical silk to a Statham force transducer. Tissue, triangles and glass holders were placed in 5 ml tissue baths containing Krebs buffer solution, aerated with 95% oxygen, 5% carbon dioxide and kept at 37°C. Free thread was connected to a Stratham Gould force transducer. One g of tension was applied to the ring. Tension was measured on a 7700 series Hewlett-Packard recorder.

The agonist (angiotensin II or epinephrine) was added cumulatively to the baths, and EC_{50} values were determined using regression analysis or the maximal contractile response calculated. The tissues were washed 5 times over a 1 h period. After washout, various concentrations of antagonists or vehicle were added and allowed at least 20 min contact with the tissues. The EC_{50} or the maximal contractile response of the agonist in the presence of antagonists or vehicle was again determined in a similar manner. The EC_{50} values in drug-treated tissues were corrected for changes in vehicle-treated tissues. The minimal effect concentrations (M.E.C.) for the compounds in antagonizing angiotensin II-induced contractile responses in the rat pulmonary artery were estimated on the basis of the drug concentration necessary to cause a $> 25\%$ reduction in the angiotensin II maximal contractile response or a ≥ 2-fold shift in the angiotensin II concentration-response curve without a change in the maximal contractile response.

The reversibility of the inhibition of angiotensin II-induced response produced by the antagonist by repeated washing of the tissues was also investigated. The contractile responses to a single concentration of angiotensin II (10 nM) estimated to produce ~ 70% of the maximal contractile response were first determined in all tissues. After washout of angiotensin II, the antagonist or vehicle was added to the baths for approximately 1 h and the tissues were rechallenged with angiotensin II. The tissues were washed 3 times every 15 min for 2.5 h and the response to angiotensin II

after washout was again determined. The degree of antagonism of angiotensin II before and after washout was corrected for changes in vehicle-treated controls.

2.5. Materials

Losartan (DuP-753), EXP3174 (2-butyl-4-chloro-1-[2′-(2*H*-tetrazol-5-yl)-1,1-biphenyl-4yl-methyl]-1-imidazole-5-carboxylate) and DuP-532 (2-propyl-4-pentafluoroethyl-1-[2′-2(*H*-tetrazol-5-yl)-1,1′-biphenyl-4-yl-methyl]-1-imidazole-5-carboxylate) were generously supplied by DuPont-Merck Pharmaceutical Company (Wilmington, DE, USA). L-163,017, an analog of a series of potent imidazopyridine antagonists previously reported (Mantlo et al., 1994) and PD121981 (WL-19) were synthesized at Merck Research Laboratories (Rahway, NJ, USA). Angiotensin II and [Sar^1,Ile^8]angiotensin II were purchased from Peninsula Laboratories (Belmont, CA, USA). ^{125}I-[Sar^1,Ile^8]Angiotensin II was purchased from DuPont-New England Nuclear (Boston, MA, USA).

3. Results

3.1. Angiotensin II radioligand receptor binding assays

In contrast to the angiotensin AT_1 receptor-selective antagonist losartan and angiotensin AT_2-selective ligand, PD121981, L-163,017 exhibited similar affinities for both angiotensin AT_1 receptor and AT_2 binding sites (Table 1). The affinity of L-163,017 (K_i = 0.11–0.20 nM) for angiotensin AT_1 receptors in rat and rabbit tissues based upon displacement of ^{125}I-[Sar^1,Ile^8]angiotensin II binding is 85–190 times greater than losartan (K_i = 11–38 nM) and 6–13 times greater than that of the active metabolite of losartan (Wong et al., 1990b), EXP3174 (K_i = 0.68–2.7 nM). L-163,017 also exhibited similar high affinity for human angiotensin AT_1 receptors expressed in CHO (Chinese hamster ovary transformed) cells (K_i = 0.14 nM) and, in the presence of a low concentration of bovine serum albumin (2 mg/ml), for angiotensin AT_1 receptors on human adrenal, aorta and kidney membranes (K_i = 0.45–3.9 nM). L-163,017 also exhibited high affinity for angiotensin AT_2 binding sites in rat adrenal and brain (K_i = 0.14 and 0.23 nM) and in human adrenal, aorta, kidney and uterus (K_i = 0.74–4.3 nM in the presence of 2 mg/ml bovine serum albumin). The much higher K_i values in some human tissues were likely due to the presence of bovine serum albumin (2 mg/ml) in the binding assay buffer since it was found that L-163,017 had K_i values of 0.13 ± 0.04 and 2.0 ± 0.14 nM in the absence and presence of bovine serum albumin in inhibiting ^{125}I-[Sar^1,Ile^8]angiotensin II binding to angiotensin AT_1 receptor in rat adrenal membranes. Moreover, L-163,017 inhibited specific ^{125}I-[Sar^1,Ile^8]-angiotensin II binding to cloned human angiotensin AT_1 receptor expressed in CHO-cell with a K_i similar to those values in rat and rabbit tissues when the binding assay buffer contained no bovine serum albumin. The affinity of L-163,017 for angiotensin AT_2 receptor (K_i = 0.14–4.3 nM) in rat, rabbit and human tissues was 7–170 times geater than PD121981 (K_i = 18–32 nM) (Table 1).

The K_i ratios (AT_2/AT_1) given in parentheses for L-163,017 are: rat adrenal (1.3), rat brain (1.8), human adrenal (1.1), human aorta (2.1) and human kidney (0.64).

In view of the report that the angiotensin II radioligand binding IC_{50} of DuP-532 (IC_{50} = 1.2 nM) was markedly affected by addition of human serum albumin (50 mg/ml) to the incubation medium (Chiu et al., 1991), IC_{50} values of the antagonists for displacement

Table 1
K_i (nM) of L-163,017, losartan, EXP3174 and PD121981 on ^{125}I-[Sar^{1}Ile^8]angiotensin II binding in various tissues

	L-163,017		Losartan		EXP3174		PD121981	
	AT_1	AT_2	AT_1	AT_2	AT_1	AT_2	AT_1	AT_2
Rat								
Adrenal	0.11 ± 0.016 [a]	0.14 ± 0.036 [b]	21 ± 1.0 [a]	> 30000 [b]	0.68 ± 0.11 [a]	> 30000 [b]	> 3000 [a]	24 [b]
Brain	0.13 ± 0.055 [a]	0.23 ± 0.088 [c]	11 ± 1.2 [a]	> 30000 [c]	0.78 ± 0.34 [a]	> 30000 [c]	> 3000 [a]	18 [b]
Rabbit								
Aorta	0.20 ± 0.004	–	38 ± 5.2	–	2.7 ± 1.6	–	> 3000	–
Human								
Adrenal [e]	3.9 ± 0.68 [a]	4.3 ± 0.47 [b]	30 [a]		36 ± 0.12 [a]	> 30000 [b]	> 3000	32 [b]
Aorta [c]	0.45 ± 0.11 [a]	0.96 ± 0.18 [b]						
Kidney [e]	3.6 ± 0.11 [a]	2.3 ± 0.59 [b]						
Uterus [e]	–	0.74 ± 0.14 [b]						
Cloned AT_1	0.14							

Values are means ± S.E.M. from at least three experiments. Values without S.E.M. are the results of one or two experiments. [a] AT_1 was determined in the presence of PD121981 (1 μM) to saturate AT_2. [b] AT_2 was determined in the presence of losartan (1 μM) to saturate AT_1. [c] Dithiothreitol (5 mM) was added to abolish AT_1 sites. [e] Bovine serum albumin (2 mg/ml) was added to the binding assay buffer.

of ^{125}I-[Sar^1,Ile^8]angiotensin II binding in the presence of a physiological concentration of human serum albumin (50 mg/ml) were determined. In the presence of human serum albumin, L-163,017 (IC_{50} = 53 ± 9.1 nM) was 8 times and 4 times more potent than losartan (IC_{50} = 400 nM) and EXP3174 (IC_{50} = 205 ± 45 nM), respectively and 100 times more potent than DuP-532 (IC_{50} = 5100 nM).

To determine whether L-163,017 interacts reversibly and competitively with angiotensin AT_1 and AT_2 receptors, ^{125}I-[Sar^1,Ile^8]angiotensin II binding to angiotensin AT_1 sites in rabbit aorta and AT_2 sites in bovine cerebellum was determined in the presence and absence of L-163,017 (1.5–2.5 nM) and analyzed according to Scatchard (1949). As shown in typical experiments in Fig. 1, L-163,017 reduced the slope and had little effect upon the x intercept of the Scatchard plot indicating a change in the K_d of angiotensin for its receptors without an appreciable change in the maximal number of receptors (B_{max}). These experiments were replicated twice with similar results. The B_{max} values were 97 ± 5% and 108 ± 1% of control values in rabbit aorta (angiotensin AT_1 receptor) and bovine cerebellum (angiotensin AT_2 receptor), respectively. The inhibition constants (K_i) for L-163,017 were estimated to be 2.5 ± 0.47 and 2.1 ± 0.2 nM at angiotensin AT_1 receptors (rabbit aorta) and angiotensin AT_2 receptors (bovine cerebellum), respectively.

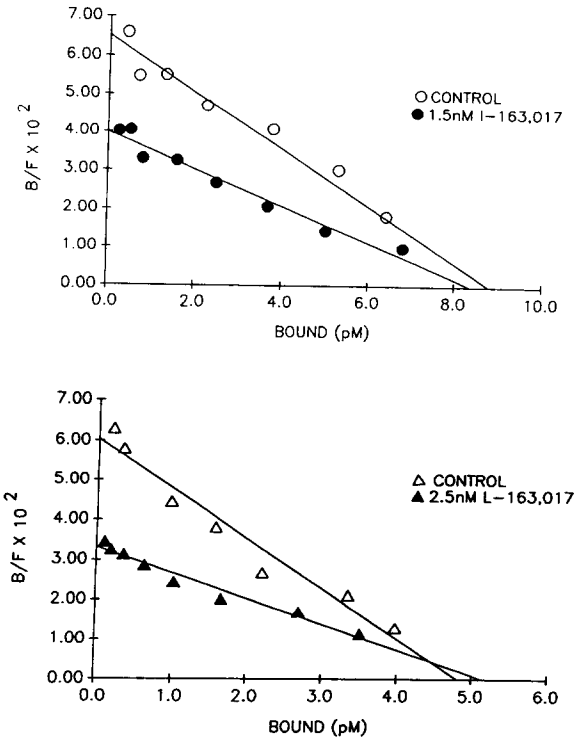

Fig. 1. Scatchard analysis of ^{125}I-[Sar^1,Ile^8]angiotensin II binding in rabbit aorta (top) and bovine cerebellum (bottom). Each point represents the means of triplicate determinations. The lines were determined by regression analysis. These experiments were replicated twice with similar results. The B_{max} values of ^{125}I-[Sar^1,Ile^8]angiotensin II binding in the presence of L-163,017 (1.5 nM and 2.5 nM for rabbit aorta and bovine cerebellum respectively) were 97±5% and 108±1% of control values in rabbit aorta and bovine cerebellum, respectively.

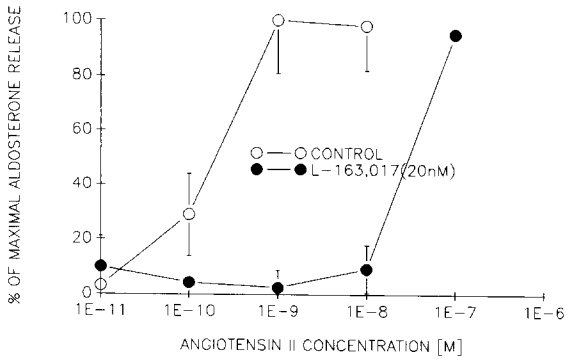

Fig. 2. Inhibition of angiotensin II-induced aldosterone release by L-163,017 in rat adrenal cells. The data points are the means ± S.E.M. of three determinations.

3.2. Other radioligand binding assays

L-163,017 (0.01–10 μM) had no appreciable effect on the binding of radioligands representative of 22 common peptide and nonpeptide receptors (see Materials and methods for the list of receptors).

3.3. Angiotensin II-induced aldosterone release from rat adrenal cells

L-163,017 (20 nM) (Fig. 2) shifted the angiotensin II dose-response curves for aldosterone release in rat adrenal cells to the right without reducing the maximal responses. Estimation of pA_2 values indicated that L-163,017 (pA_2 = 9.9, K_b = 0.13 nM) was 100 times more potent than losartan (pA_2 = 7.9, K_b = 13 nM) in antagonizing angiotensin II-induced aldosterone release in this preparation. L-163,017 (20 nM), alone, had no effect upon aldosterone release indicating a lack of agonist activity.

3.4. Angiotensin II-induced contraction in isolated rat pulmonary artery

L-163,017 (0.10–1.0 nM) antagonized the contractions produced by angiotensin II in the pulmonary artery as a function of concentration (Fig. 3). At these concentrations, L-163,017 alone did not affect the resting tension of the tissue indicating a lack of angiotensin AT_1 agonist activity. The antagonism of angiotensin II produced by L-163,017 (0.10–1.0 nM) was characterized by a shift to the right of the angiotensin II concentration-response curve which was associated with a significant reduction in the maximal contractile re-

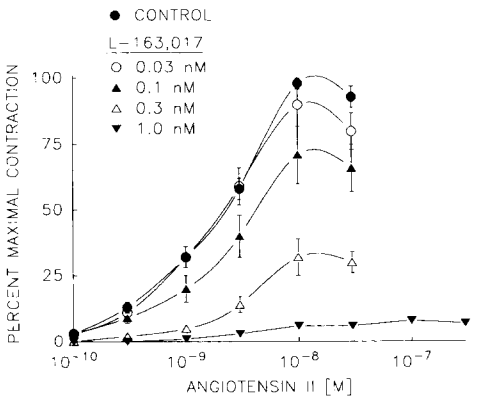

Fig. 3. Antagonism of angiotensin II-induced contraction by L-163,017, in isolated rat pulmonary artery. The data points are means ±S.E.M. of at least three determinations.

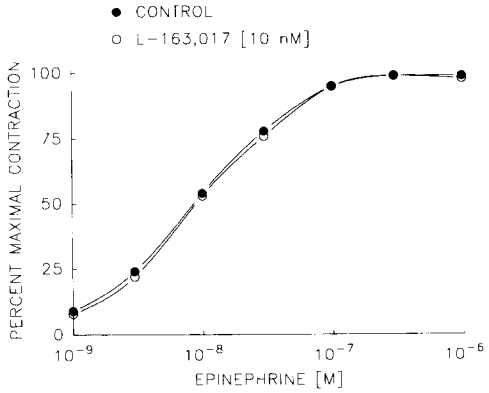

Fig. 5. Lack of effect of L-163,017 upon epinephrine-induced contraction of the isolated pulmonary artery. The data points are means ±S.E.M. of at least three determinations.

sponse (Fig. 3). These results were similar to those reported previously from this laboratory for EXP3174 and MK-996 but were different from losartan which produced a parallel shift to the right of the angiotensin II concentration-response curves without a reduction in the maximal contractile response (Chang et al., 1994). Comparison of the estimated minimal effective concentration (M.E.C.) of the compounds for antagonism of angiotensin II contractions indicated that L-163,017 (M.E.C. = 0.1 nM) has a similar potency as EXP3174 (M.E.C. = 0.1 nM) and approximately 40 times greater than losartan (M.E.C. = 4.2 nM).

The reduction in the angiotensin II maximal contractile response produced by L-163,017 and EXP3174, but not losartan, in vascular tissues could be the result of a slower dissociation rate of the former compounds from the angiotensin II receptor. Studies were conducted to determine whether this might be reflected in the relative resistance of the compound to being removed from the tissues by repeated washing. As shown in Fig. 4, repeated washing of the tissues did not affect the antagonism of angiotensin II produced by L-163,017, but essentially completely reversed the angiotensin II antagonism produced by losartan (DuP-753) and EXP3174. The data suggested that L-163,017 exhibited a slower dissociation rate than losartan and EXP3174. However, differences in the dissociation rates of losartan and EXP3174 were not detectable using these methods. The differences in the pattern of the angiotensin II antagonism produced by L-163,017 compared to losartan in the rat pulmonary artery thus could be the result of a slower rate of dissociation of L-163,017 from the angiotensin II receptor than losartan.

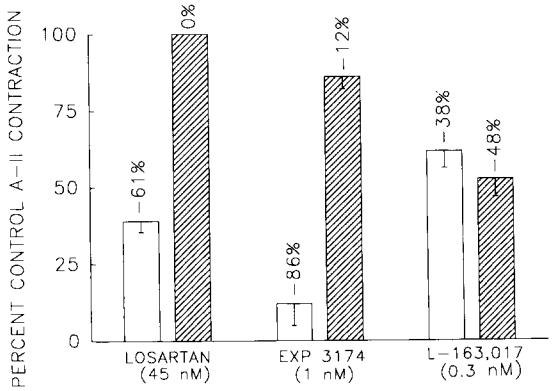

Fig. 4. In vitro washout of angiotensin II antagonists in the isolated rat pulmonary artery. The contractile responses to a single concentration of angiotensin II (10 nM) were first determined in all tissues. After washout of angiotensin II, tissues were treated with vehicle or antagonists for approximately 1 h. Open columns: after antagonist treatment, tissues were rechallenged with angiotensin II; shaded columns: after antagonist treatment and angiotensin II rechallenge, tissues were washed 3 times at 15 min intervals for 2.5 h before the angiotensin II rechallenge. Values on top of each column are means of percent of inhbition of angiotensin II response by losartan, EXP3174 and L-163,017 before and after washout from at least three determinations. The degree of antagonism of angiotensin II before and after washout was corrected for change in vehicle-treated group.

L-163,017 demonstrated specificity for antagonism of contractions produced by angiotensin II in the rat pulmonary artery compared with another contractile agonist of this tissue. Concentrations of L-163,017 (10 nM) which were highly effective for antagonism of angiotensin II, did not significantly affect the concentration-response curves or maximal contractile responses to epinephrine in the pulmonary artery (Fig. 5).

4. Discussion

In contrast to the angiotensin AT_1- or AT_2-selective antagonists, L-163,017 exhibits similar high affinities for both angiotensin AT_1 and AT_2 binding sites in various tissues from rat, rabbit and human. The ratios of K_i values for L-163,017 in inhibiting specific angiotensin AT_2 and AT_1 binding sites in a given tissue

known to contain both two receptors are 2 or less. L-163,017 is 85–190 times more potent (in the absence of bovine serum albumin) than losartan and 6–13 times more potent than the losartan metabolite, EXP3174 on the angiotensin AT_1 receptor.

To determine whether L-163,017 interacts reversibly and competitively with angiotensin AT_1 and AT_2 receptors, ^{125}I-[Sar^1,Ile^8]angiotensin II binding to angiotensin AT_1 sites in rabbit aorta and AT_2 sites in bovine cerebellum was determined in the presence and absence of L-163,017 (1.5–2.5 nM) and analyzed according to Scatchard. As shown in Fig. 1, L-163,017 reduced the slope and had little effect upon the x intercept of the Scatchard plot indicating a change in the K_d value of angiotensin for its receptors without an appreciable change in the maximal number of receptors. The inhibition constants (K_i) for L-163,017 were estimated to be 2.5 ± 0.47 and 2.1 ± 0.2 nM at angiotensin AT_1 receptors (rabbit aorta) and AT_2 receptors (bovine cerebellum), respectively. The data are consistent with L-163,017, interacting in a reversible and competitive manner with angiotensin AT_1 and AT_2 receptors.

Functional in vitro studies were conducted to characterize the nature of the interaction of L-163,017 with the angiotensin II receptor. L-163,017 antagonized the increase in aldosterone release induced by angiotensin II in the rat adrenal cells. In this assay, L-163,017 produced a parallel shift in the angiotensin II concentration-response curve without changing the maximal response. The result is consistent with a competitive reversible interaction of L-163,017 with the angiotensin II receptor (Taylor and Insel, 1990) and confirms the data obtained using Scatchard analysis of the binding data in the rabbit aorta. The estimated pA_2 for L-163,017 as an antagonist of angiotensin II-induced aldosterone release ($pA_2 = 9.9$, $K_b = 0.13$ nM) was also in agreement with the K_i value (0.11 nM) obtained in the radioligand binding studies.

L-163,017 additionally antagonized the contractile responses to angiotensin II in the isolated rat pulmonary artery. In this assay, L-163,017 produced a shift to the right in the angiotensin II concentration-response curve which was associated with a significant reduction in the maximal response to angiotensin II. Such a pattern of antagonism can be observed with competitive antagonists which are irreversibly (covalently bond) or pseudoirreversibly (slowly dissociable) associated with receptors, as well as with noncompetitive antagonists (Taylor and Insel, 1990). Considering that L-163,017 behaved in a competitive and reversible manner in radioligand binding studies and as an antagonist of angiotensin II-induced aldosterone release, it is most likely that the reduction in angiotensin II maximal response in these assays is due to slowly dissociable, albeit competitive, antagonism. In support of this view, a major difference between the radioligand and aldosterone studies compared to the vascular contraction studies is that in the former assays angiotensin II and L-163,017 were coincubated, whereas the latter assay involved preincubation with L-163,017 before addition of angiotensin II. The time for an agonist to achieve equilibruim with the receptor is inherently longer when the receptors are already in equilibrium with a slowly dissociable antagonist. It is possible that the reduction in the angiotensin II maximal response observed in the L-163,017 preincubation studies is due to the inability of angiotensin II to achieve equilibrium with the receptor within the time constraints of these experiments because of a slow dissociability of L-163,017. The slow dissociation or irreversible action of L-163,017 from angiotensin II receptor was directly supported by the experiments showing that repetitive washing for 2.5 h failed to reverse the angiotensin II response following L-163,017 antagonism in isolated pulmonary artery.

In the absence of angiotensin II, L-163,017 did not stimulate aldosterone release nor did it cause contraction of the vascular tissues demonstrating a lack of agonist activity. Moreover, L-163,017 demonstrated specificity for angiotensin II response since it did not affect the response to other agonists in the functional in vitro assay and was inactive in many radioligand binding assays.

Since both angiotensin II-induced aldosterone release in rat adrenal cells and angiotensin II-induced contractile response in isolated rat pulmonary artery are mediated by angiotensin AT_1 receptors (Chang and Lotti, 1989, 1990; Chang et al., 1992), the antagonism of angiotensin II responses by L-163,017 in these assays must be contributed by its affinity for angiotensin AT_1 receptors. Hence, no functional study was performed in the present study to determine that L-163,017 was an agonist or antagonist on angiotensin AT_2 receptor. In this regard, it was recently reported that a small angiotensin II-induced inhibition of phosphotyrosine phosphatase in the cells expressing cloned angotensin AT_2 receptors or a cell line (PC12W) containing only angiotensin AT_2 receptors was antagonized by another angiotensin AT_2 ligand, PD123319, but not by losartan, an angiotensin AT_1-selective antagonist (Kambayashi et al., 1993). The activity of L-163,017 in these systems remained to be determined.

In summary, the present data demonstrate that L-163,017 is a highly potent competitive and specific antagonist of angiotensin AT_1 receptors in in vitro radioligand binding and functional isolated tissue assays. L-163,017 has similar high affinity for the angiotensin AT_2 receptor and is also found to be orally effective and to have long duration in animals (Kivlighn et al., 1995b). Moreover, the plasma bioequivalencies for angiotensin AT_1 and AT_2 receptors determined

using the radioreceptor assays indicated that L-163,017 maintained balanced activity for angiotensin AT_1 and AT_2 receptors for at least 8 h following i.v. or p.o. administration to rat or dog (Kivlighn et al., 1995b). These properties make L-163,017 a new tool for investigating the physiological and pharmacological action of angiotensin II. L-163,017 may also have therapeutic potential as it possesses the necessary attributes for a development candidate.

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