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BMS-180560, an insurmountable inhibitor of angiotensin II-stimulated responses: comparison with losartan and EXP3174

[1]K.E.J. Dickinson, R.B. Cohen, S. Skwish, C.L. Delaney, R.P. Serafino, M.A. Poss, Z. Gu., D.E. Ryono, S. Moreland & J.R. Powell

Departments of Cardiovascular Biochemistry, Pharmacology, and Chemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543-4000, U.S.A.

1 This study compares the activity of BMS-180560 (2-butyl-4-chloro-1-[[1-[2-(2H-tetrazol-5-yl)phenyl]-1H-indol-4-yl]methyl]-1H-imidazole-5-carboxylic acid), an insurmountable angiotensin II (AII) receptor antagonist, with that of losartan and EXP3174 in functional and biochemical models of AII-receptor activation.

2 BMS-180560 selectively inhibited [^{125}I]-Sar^{1}Ile^{8}AII ([^{125}I]SI-AII) binding to rat aortic smooth muscle (RASM) cell and rat adrenal cortical AT_1 receptors ($K_i = 7.6 \pm 1.2$ and 18.4 ± 3.9 nM respectively) compared to adrenal cortical AT_2 receptors ($K_i = 37.6 \pm 1.3$ μM). The K_i values of BMS-180560 and EXP3174, but not losartan, varied as a function of the BSA concentration used in the assays, indicating that the diacid drugs bound to albumin.

3 BMS-180560 (3–300 nM) increased the K_D of SI-AII for RASM cell AT_1 receptors. Only at high concentrations of BMS-180560 (300 nM) were B_{max} values decreased.

4 BMS-180560 inhibited AII-stimulated contraction of rabbit aorta with a calculated $K_B = 0.068 \pm 0.048$ nM and decreased maximal AII-stimulated contraction at 1 nM BMS-180560 by 75%. In the presence of 0.1% BSA, a higher K_B value (5.2 ± 0.92 nM) was obtained. Losartan behaved as a competitive antagonist with a $K_B = 2.6 \pm 0.13$ nM. Contraction stimulated by endothelin-1, noradrenaline, KCl, or the TXA_2 receptor agonist U-46619 were unaffected by BMS-180560 (1 nM).

5 AII stimulated the acidification rates of RASM cells as measured by a Cytosensor microphysiometer with an EC_{50} of 18 nM. Losartan (30 nM) shifted the AII concentration-effect curves in a competitive manner whereas BMS-180560 (0.01 and 0.1 nM) decreased the maximum responses by 60 and 75% respectively. Inhibition by losartan and BMS-180560 could be reversed following washout although recovery took longer for BMS-180560.

6 In [^{3}H]-myoinositol-labelled RASM cells, losartan (30 and 200 nM), shifted the EC_{50} for AII-stimulated [^{3}H]-inositol monophosphate formation to higher values, with no change in the maximal response. By contrast, EXP3174 (0.1 to 1 nM) decreased the maximal response in a concentration-dependent manner (17–55%). BMS-180560 (3 and 10 nM) increased the EC_{50} for AII and decreased the maximum response by 30 and 80% respectively. The inhibition by EXP3174 and BMS-180560 could be reversed by inclusion of losartan (200 nM) indicating that the inhibition was not irreversible.

7 In conclusion, BMS-180560 is a potent, specific, predominantly competitive, reversible AII receptor antagonist, which displays insurmountable receptor antagonism. At concentrations of BMS-180560 which have no effect on receptor number, BMS-180560 produced insurmountable antagonism of AII-stimulated second messenger formation, extracellular acidification, and smooth muscle contraction.

Keywords: Angiotensin II receptor; vascular smooth muscle; [^{125}I]-Sar^{1}Ile^8 binding; adrenal cortex; insurmountable antagonism; losartan; BMS-180560

Introduction

The antiotensin II (AII) receptor contributes significantly to hypertension in man and therefore represents a key target for the design of anti-hypertensive agents. Recently a number of non-peptidic AII receptor antagonists have been described which provide a new class of anti-hypertensive agents (Timmermans *et al.*, 1991), and indicate the potential for small molecules to mimic the binding of an octapeptide to its receptor. The use of these non-peptide antagonists has provided evidence for subtypes of AII receptors (Chiu *et al.*, 1989; Whitebread *et al.*, 1989; Chang & Lotti, 1990). Thus, AII receptors which exhibit high affinity for losartan are designated AT_1 receptors, the classical AII receptors which modulate vascular tone and adrenal aldosterone secretion. The AT_1 receptor is coupled in most tissues via a G protein to phospholipase C activation, inositol phospholipid breakdown, and Ca^{2+} mobilization (Smith *et al.*, 1984; Griendling *et al.*, 1987; 1991). This receptor therefore represents the target site for potential anti-hypertensive and anti-hypertrophic agents. AT_2 receptors exhibit low affinity for losartan but high affinity for PD 123,177 (Wong *et al.*, 1990a; Dudley *et al.*, 1990). Both AT_1 and AT_2 receptors are present in different proportions on membranes derived from rat adrenal cortex and medulla (Chang & Lotti, 1990), whereas rat phaeochromocytoma PC-12W cells contain a homogeneous population of AT_2 receptors (Speth & Kim, 1990). The function of AT_2 receptors on PC12W cells (Webb *et al.*, 1992b) or other cells (Pucell *et al.*, 1991; Dudley *et al.*, 1991) is unknown.

Peptide analogues of AII have been described which function as antagonists of AII-mediated responses. Thus, Phe^4,Tyr^{8}AII exhibits classical competitive receptor antagonism and produces parallel shifts of the AII-stimulated

[1] Author for correspondence at Dept. of Cardiovascular Biochemistry.

concentration-response curves of smooth muscle preparations without suppressing the maximum response (Pendleton *et al.*, 1989). By contrast, sarcosyl substituted AII peptides including Sar^1,Leu^8AII inhibit AII-mediated contraction of rabbit aorta in an insurmountable manner; the maximum AII-stimulated contraction cannot be overcome by increasing concentrations of AII (Pendleton *et al.*, 1989; Liu *et al.*, 1992). The basis of this activity has been attributed to slow dissociation of these peptides from the AII receptor which results in a pseudo-irreversible inhibition (Pendleton *et al.*, 1989) or to ligand-mediated decreases in available AII receptors (Liu *et al.*, 1992).

A number of nonpeptidic AII receptor antagonists exhibit classical receptor antagonism. These include the earliest known examples of nonpeptide antagonists such as S-8307 (Wong *et al.*, 1988), and more recent disclosures such as losartan (Wong *et al.*, 1990a,c), SC 51366 (Olins *et al.*, 1992) and SKF 108566 (Edwards *et al.*, 1992). Schild slopes of antagonists such as losartan are close to unity (Wong *et al.*, 1990a), which indicates that the interaction of losartan with the AT_1 receptor represents a reversible, competitive reaction. Nonpeptidic AII receptor antagonists have also been described which display insurmountable inhibition of AII-stimulated contraction of vascular preparations. These include EXP3174, the di-acidic metabolite of DuP 753 (Wong *et al.*, 1990b), EXP3892 (Wong & Timmermans, 1991), DuP 532 (Wong *et al.*, 1991), L-158,809 (Chang *et al.*, 1992), SR 47436 (Cazaubon *et al.*, 1993) and GR 117289 (Robertson *et al.*, 1992). It is unlikely that the insurmountable behaviour relates to irreversible binding of antagonists since losartan has been shown to attenuate the EXP3174-induced decrease in maximum AII-stimulated contractions (Wong & Timmermans, 1991). The basis of this activity is currently unknown but it may contribute to the long duration of action of these compounds *in vivo*. No direct evidence for the location of the sites responsible for insurmountable antagonism have so far been presented although most models implicate the AII receptor. The present paper characterizes the pharmacological properties of BMS-180560, a di-acidic AII receptor antagonist which produces long-lasting anti-hypertensive effects in Na^+-depleted SHR, and potently antagonizes AII pressor responses in conscious Sprague Dawley rats (Poss *et al.*, 1993). The present study compares the interaction of BMS-180560, losartan and EXP3174 with AT_1 receptors using functional contraction measurements, binding techniques, and biochemical determinations of AII-receptor activation. A preliminary account of these findings was presented to the British Pharmacological Society (Dickinson *et al.*, 1992).

Methods

Membrane preparation

Rat adrenal cortex Rats were killed by CO_2 asphyxiation, the adrenals were removed, and the cortex separated from the medulla at 4°C. The cortices were placed in ice-cold buffer A (0.2 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4), and homogenized in 10-volumes of buffer A at 4°C with a Brinkman Polytron homogenizer (setting 9.3 × 8 s). The homogenate was centrifuged at 1000 *g* for 10 min at 4°C, the supernatant was re-centrifuged at 100,000 *g* for 1 h at 4°C. The pellet was suspended in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ (2.5 vol of buffer/original wet weight), by gentle homogenization in a Potter-Elvehjem glass/teflon homogenizer. Aliquots (5 ml) of the membrane preparation were frozen at a protein concentration of 0.1 mg protein ml^{-1} and stored at −80°C until use.

Rat aortic smooth muscle cells RASM cells were cultured at 37°C in T-75 flasks under humidified 95% air/5% CO_2 in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 50 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (Gibco Labs). Following the attainment of confluence (5–7 days), cells were trypsinized with 2 ml 0.25% trypsin/1 mM EDTA and cells collected into buffer (0.1 mM phenylmethylsulphonyl fluoride, 10 μg ml^{-1} soy bean trypsin inhibitor, 20 mM HEPES pH 7.4, dissolved in DMEM) at a concentration of $3–4 \times 10^5$ cells ml^{-1}. The cell suspension was washed with this buffer, and homogenized in 50 mM Tris HCl pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 0.24 TI units ml^{-1} aprotinin, and 0.1 mg ml^{-1} 1,10-phenanthroline with a Brinkmann Polytron homogenizer (setting 7, 3 × 6 s). The homogenate was passed through 2 layers of cheesecloth, and centrifuged at 40,000 *g* for 20 min at 4°C. The supernatant was discarded, and the membranes resuspended in buffer and washed three times. The pellet was resuspended in this buffer at a concentration of 0.2–0.8 mg protein ml^{-1}. The cell homogenate was stored in 1 ml aliquots at −80°C until use.

[^{125}I]-Sar^1,Ile^8 angiotensin II binding

Assays were conducted in a total volume of 250 μl in tubes arranged in microtitre plate format (Marsh Biomed Corp). The incubation mixture contained 50 μl [^{125}I]-SI-AII (80–200 pM, 70,000–180,000 c.p.m.); 25 μl displacing drug, or AII to define non-specific binding (1 μM); and incubation buffer. Binding to RASM cell membranes was conducted in the following assay buffer: 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1% bovine serum albumin, 1 mM PMSF, 0.24 TI units ml^{-1} aprotinin, and 0.1 mg ml^{-1}, 10-phenanthroline (Cohen *et al.*, 1993). Binding to rat adrenal cortex (RAC) membranes was routinely conducted in the following assay buffer: 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, and 0.22% BSA. In some cases the concentration of BSA was changed to 0.07%, or 0.01%. The binding reaction was initiated by the addition of 100 μl membranes (7–12 μg protein) diluted in incubation buffer. The tubes were incubated at 37°C (RASM cell membranes) or 22°C (RAC membranes) for 2 h with continuous shaking (Easyshaker, SLT-Labinstruments, A-5082 Grodig, Austria). Bound and free radioligand were separated by simultaneous filtration on a Tomtec cell harvester in combination with a filtermat B which had been pre-soaked for 1 h in 0.1% polyethyleneimine (PEI) in order to reduce non-specific binding. The filtermat was rinsed of excess PEI during a pre-wash cycle, and the membranes were filtered and washed with 150 mM NaCl, 5 mM Tris-HCl, pH 7.4 at 4°C. The filtermat was removed and microwaved, membrane-side up, at full power for 3 × 2 min in a microwave oven. The dried filtermat was placed in a sample bag, a sheet of Meltilex solid scintillant wax placed on the filtermat and the Meltilex sheet melted into the mat using a T-Tray Heat-Sealer (Wallac, Pharmacia). The impregnated sheet was counted in a Betaplate liquid scintillation counter (L.K.B. Pharmacia, T-tray compatible Model 1205) at 60% efficiency. Protein assays were performed on the membrane preparation using B.C.A. reagent (Pierce, Rockford, IL) with BSA as standard.

Measurements of cell activation using Cytosensor microphysiometer

The Cytosensor microphysiometer (Molecular Devices, Menlo Park, CA, U.S.A.) measures extracellular acidification rates of cells with silicon pH-sensitive sensors (Owicki *et al.*, 1990). Upon activation of cells with agonists, the extracellular acidification rate increases in a time and concentration-dependent manner. RASM cells were cultured for 8 h in the presence of foetal bovine serum (10%), the serum was removed and after a further 16 h cells were placed in the microphysiometer and perfused with media (Dulbeccos MEM without $NaHCO_3$) at 37°C for 1 h prior to drug addition. Acidification rates were monitored for 30 s every min and rates plotted continuously against time. RASM cells

were stimulated with the stated concentrations of AII for 10 s followed by a change of perfusate to media. Short periods of AII application were necessary because longer stimulation protocols resulted in desensitized responses. Using this protocol, peak increases in acidification rates were obtained in response to AII. AII was added every 15 min to a maximum concentration of 10 μM. Antagonists BMS-180560 and losartan were preincubated with cells for 15–30 min prior to AII addition. Peak height was used as a measure of the cellular activation. Maximum responses to AII routinely represented 130% of basal acidification rate (normalized to be 100%). Results reported are the means (± s.e.mean) of at least three determinations with different passage numbers.

Measurement of phosphoinositide turnover

RASM cells were cultured in 35 mm wells, and labelled to isotopic equilibrium with [^{3}H]-myoinositol (2 μCi ml^{-1}) in inositol-free medium for 48 h. Cell monolayers (approximately 90% confluent) were washed, and incubated for 15 min at 37°C in a medium containing 10 mM LiCl in the absence or presence of AII antagonist. The cells were stimulated with agonist for 30 min, the media removed, and 2 mM EDTA at 100°C was added to the cell monolayer to disrupt cell integrity and release soluble inositol phosphates (IP). The cells and supernatant were removed, re-boiled, and centrifuged. The supernatant was applied to a Dowex AG-1X8 anion exchange column, the labelled inositol, glycerophosphoinositol, and inositol 1-, 2-, and 3-phosphates fractionated essentially as described by Berridge (1983) and aliquots counted in a liquid scintillation counter using Packard Ultima Gold XR scintillation fluid (Packard Inst. Co., Meriden, CT, U.S.A.).

Force determinations in vascular smooth muscle

Male New Zealand white rabbits (2–3 kg) were killed by i.v. injection of Nembutal and the thoracic aorta was removed and cleaned of connective tissue. Circumferential strips (3.5–5.0 mm wide) were mounted for isometric force recording as described in detail elsewhere (Webb *et al.*, 1992a). In brief, cumulative concentration-response curves were elicited by agonists (AII, ET-1, KCl, noradrenaline, and U-46619) in the presence and absence of test compound. Antagonists were preincubated with the tissue for 20 min prior to addition of agonists. The data are plotted as the mean (± s.e.mean) of at least 4 tissues from different animals. EC_{50} values were calculated by linear regression analysis. K_B values for competitive antagonists were calculated from the following equation: $K_B = BMS/[EC_{50}'/EC_{50})-1]$, where BMS represents the concentration of compound tested, EC_{50}' is the EC_{50} value determined in the presence of BMS, and EC_{50} is the EC_{50} value determined in response to AII alone. For insurmountable antagonists, the $K_{B\ app}$ values were calculated by double-reciprocal regression analysis: $K_{B\ app} = BMS/(slope-1)$ where the slope is obtained by regression analysis of 1/[A] versus 1/[A'] with [A] and [A'] denoting the equiactive agonist concentrations in the absence and presence of BMS, respectively (Tallarida & Jacob, 1979).

Drugs

[^{125}I]-Sarcosine^{1}Ile^{8}angiotensin II ([^{125}I]-SI-AII, 2200 Ci mmol^{-1}) was obtained from NEN Research Products (Boston, MA, U.S.A.), and [^{3}H]-myoinositol (94 Ci mol^{-1}) was from Amersham (Arlington Hts, IL, U.S.A.). Angiotensin II and SI-AII were from Peninsula Labs (Belmont, CA, U.S.A.), and cell culture reagents from Gibco (Rockville, MD, U.S.A.). Bovine serum albumin, lithium, sodium, magnesium and potassium chlorides, noradrenaline, sucrose, EDTA, EGTA, phenylmethylsulphonylfluoride (PMSF), polyethyleneimine, aprotinin, 1,10-phenanthroline, HEPES, and Tris were from Sigma Chemical Co (St. Louis, MO, U.S.A.). Dowex AG-1X8 was from Biorad (Richmond, CA, U.S.A.). U-46619 (11α, 9α-epoxymethano-PGH$_2$) was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.) and endothelin-1 from Peptides International (Louisville, KY, U.S.A.). All other chemicals were from Fisher (Pittsburgh, PA, U.S.A.) and were reagent grade. Losartan, EXP3174 and BMS-180560 were synthesized in Department of Chemistry, Bristol-Myers Squibb Pharmaceutical Research Institute.

Results

Figure 1 shows the structures of losartan, its metabolite EXP3174 and BMS–180560 which differs from the biphenyl compounds losartan and EXP3174 in having an indole linker between the imidazole and phenyl tetrazole moieties. The chemical synthesis of BMS-180560 and the *in vivo* pharmacological activity of BMS-180560 and its prodrugs are described elsewhere (Poss *et al.*, 1994).

Binding studies

The ability of losartan and BMS-180560 to inhibit [^{125}I]-SI-AII binding to rat adrenal cortical, and aortic smooth muscle cell membranes was compared. Figure 2 shows the competition curves, and Table 1 shows the K_i values obtained under standard assay conditions. Competition curves for losartan and BMS-180560 binding to adrenal AII receptors were biphasic and indicative of binding to two populations of sites (Figure 2a). A high affinity population (AT_1 receptors), and a low affinity population (AT_2 receptors) represented 75% and 25% of the labelled sites respectively. Both losartan and BMS-180560 were selective for the AT_1 receptors relative to the AT_2 receptors (9,000 and 2,000 fold respectively). Both antagonists generated competition curves with RASM cell membranes which were monophasic, with slope factors which were close to unity indicating a homogeneous, high affinity, population of AT_1 receptors (Figure 2b, Table 1). The K_i values obtained for losartan and BMS-180560 for vascular

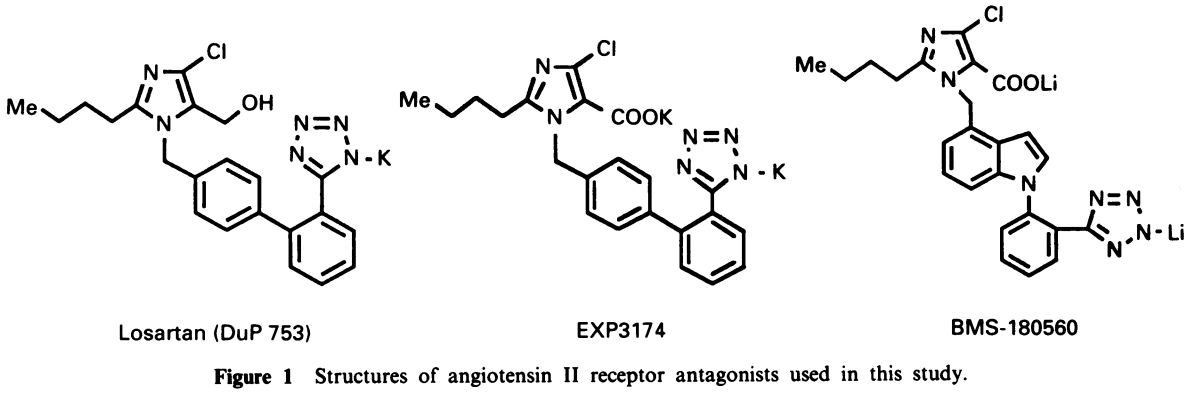


Figure 1 Structures of angiotensin II receptor antagonists used in this study.

Table 1 Inhibition constants (K_i) of losartan and BMS-180560 for rat adrenal and smooth muscle cell AII receptors

Compound	K_i values (nM) Adrenal AT_1	Adrenal AT_2	RASM cell AT_1	n_H
Losartan	9.0 ± 3.2*	85,800 ± 8.700*	16.2 ± 4.6	0.91 ± 0.03
BMS 180560	18.4 ± 3.9	37,600 ± 13,000	7.6 ± 1.2	0.99 ± 0.17

n_H = slope factor of competition curves for RASM cell binding. *Slope factor constrained to $n_H = 1$ for two-site analysis, which yielded the proportions of AT_1 and AT_2 sites of 75 ± 1, and 25 ± 1% respectively. K_i values were computed from IC_{50} values using the equation of Cheng & Prusoff (1973). Results show mean (±s.e.mean) for 3–10 experiments.

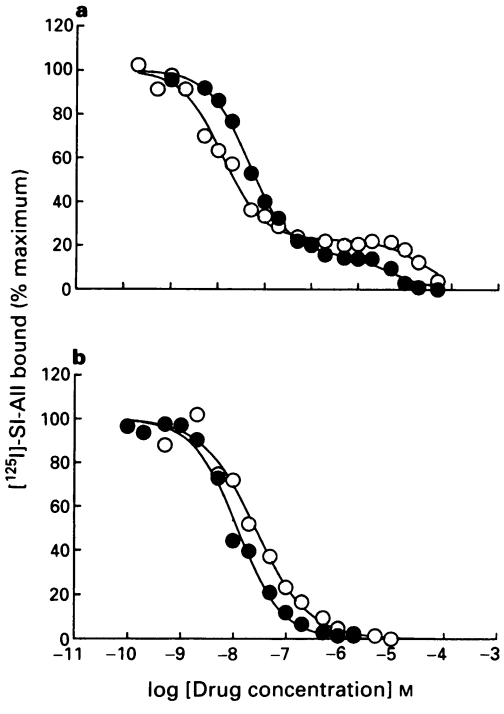


Figure 2 Competition curves of losartan (○) and BMS-180560 (●) binding to rat adrenal cortical (a) and RASM cell (b) membranes. Membranes were incubated with [^{125}I]-SI-AII (0.2 nM) in the presence of the shown concentrations of antagonists. Specific binding, calculated as % maximum, has been plotted against antagonist concentration. The lines are computer generated best fit to a one or two site analysis where slope factors for both sites are constrained to unity. The curves are representative of experiments performed 3–10 times.

AII receptors (16 and 18 nM) were similar to those obtained for adrenal cortical receptors (9 and 18 nM).

Recent reports indicate diacid AII receptor antagonists bind avidly to serum proteins, whereas losartan show less activity in this regard (Chiu *et al.*, 1991). Since this property reduces the free drug able to interact with the AII receptor, we have investigated the influence of BSA concentration on the calculated K_i of BMS-180560. Table 2 shows that reduction of BSA in the assay buffer from 0.22 to 0.07% decreased the K_i of BMS-180560 for adrenal AT_1 receptors by 3.4 fold. Decreasing the BSA to lower values (0.01%) decreased the K_i by a further 6.8 fold to a value of 0.8 nM. Similar results were obtained for EXP3174 which exhibited a K_i of 11.4 ± 2.6 nM in 0.22% BSA and 1.9 ± 1.4 nM in 0.07% BSA ($n = 4$). Losartan was insensitive to the BSA concentration in the assay over the concentration-range tested, and K_i values obtained with 0.01% BSA were not significantly different from those determined in 0.22% BSA (Table 2). Similar findings were observed with RASM membranes, where the K_i for losartan was 17 ± 1.4 nM ($n = 3$) in 0.01% BSA, and 16.2 ± 4.6 nM ($n = 3$) in 0.1% BSA.

The characteristics of losartan and BMS-180560 binding to the rat vascular smooth muscle cell AT_1 receptors were compared. Saturation binding isotherms were conducted in the absence or presence of increasing concentrations of losartan or BMS-180560, and K_D and binding site maxima (B_{max}) values calculated in order to define shifts in affinity (competitive inhibition) or decreases in B_{max} values, with no change in K_D (non-competitive inhibition). Time course studies indicated that equilibrium binding of [^{125}I]-SI-AII was reached within 120 min either in the absence or presence of 3–30 nM BMS-180560 (data not shown). In order to obtain accurate assessment of the B_{max} values we have used tracer concentrations of radioligand and saturated the binding sites with unlabelled SI-AII. Data analysis assumed that tracer radioligand and SI-AII bind to the same population of sites with similar affinities. This assumption is valid since K_D and B_{max} values using exclusively [^{125}I]-SI-AII ($K_D = 0.3 \pm 0.04$ nM, $B_{max} = 2{,}860 \pm 700$ fmol mg^{-1} protein, $n = 4$) were similar to values reported in Table 3.

Table 2 Effect of BSA concentration on the inhibition constants of BMS-180560 and losartan for rat adrenal cortical AT_1 receptors

Drug	K_i values (nM) 0.01% BSA	0.07% BSA	0.22% BSA
BMS-180560 AT_1	0.8 ± 0.1	5.4 ± 2.0	18.4 ± 3.9
Losartan AT_1	9.8 ± 0.1	NT	9.0 ± 3.2

K_i values (nM) were calculated from competition curves determined in the presence of the stated concentration of BSA. Results show mean ± s.e.mean K_i values of drugs for AT_1 receptors derived from 3–6 experiments.

The presence of losartan resulted in shifts to the right of the SI-AII saturation binding isotherms with progressive increases in K_D values (see Table 3), but no decreases in the maximum number of binding sites. Figure 3a shows the Scatchard plots of representative data where losartan caused successive decreases in the slopes (indicative of increased K_D values for SI-AII), with no decreases in the binding site maxima (defined by the lines' intercept with the abscissa). These data are consistent with reversible competitive interaction of losartan with the AT_1 receptor. These data were analysed by the method of Arunlakshana & Schild (1959) in order to define a binding K_B value for losartan. The calculated Schild slope was 1.07 ± 0.06 and the K_B was 10 ± 2.9 nM, which is in good agreement with the K_i of 16.2 nM determined from competition curves.

BMS-180560 (3–100 nM) also shifted the SI-AII saturation binding isotherms to the right and increased the K_D of SI-AII. This is shown in Figure 3b as a successive decrease in the slopes of the Scatchard plots. At low concentrations of BMS-180560 (3–30 nM), there was no significant depression of the B_{max} values (Table 3), which indicated that the drug acted as a competitive inhibitor of SI-AII binding. Similar results were obtained with membranes either pre-incubated with BMS-180560 for 30 min or co-incubated with the antagonist (data not shown). At a higher concentration of BMS-180560 (100–300 nM), there was a 10 to 20 fold in-

crease in the K_D for SI-AII, and a decrease in the B_{max} value at the highest BMS-180560 concentration. Similar results were obtained using exclusively [^{125}I]-SI-AII to saturate the binding sites (data not shown). These data suggest that BMS-180560 interacts competitively at low concentrations but may exhibit mixed (competitive and non-competitive) inhibition at higher concentrations. The Schild slope of these binding data which also incorporates the higher drug concentrations was 0.89 ± 0.06, and the calculated K_B value from this plot was 9.0 ± 4.6 nM, which is in good agreement with the K_i of 7.6 nM calculated from competition curves.

Table 3 Effect of losartan and BMS-180560 on SI-AII binding to RASM cell membranes

Drug	*Conc.* (nM)	B_{max} (% control)	n	K_D (nM)	n
Losartan	0	100		0.20 ± 0.04	4
	5	110 ± 12	3	0.28 ± 0.04	3
	20	119 ± 10	4	0.84 ± 0.33	4
	50	121 ± 9	3	1.06 ± 0.27	3
BMS-180560	0	100		0.20 ± 0.04	4
	3	91	2	0.32	2
	10	97 ± 3	3	0.39 ± 0.05	3
	30	91 ± 3	5	0.86 ± 0.05	5
	100	88 ± 7	3	2.13 ± 0.25	3
	300	53 ± 8	3	4.0 ± 0.10	3

RASM membranes were pre-incubated for 15 min at 37°C with the stated concentrations of antagonists, and incubated with ligand [^{125}I]-SI-AII (0.2 nM) in the presence of increasing concentrations of SI-AII. Specific binding was calculated and analysed by computer assisted iterative curve fitting to obtain B_{max} and K_D values. B_{max} values have been normalized to % control which was $2,840 \pm 430$ fmol mg^{-1} protein. Results show mean ± s.e.mean of the stated number of experiments.

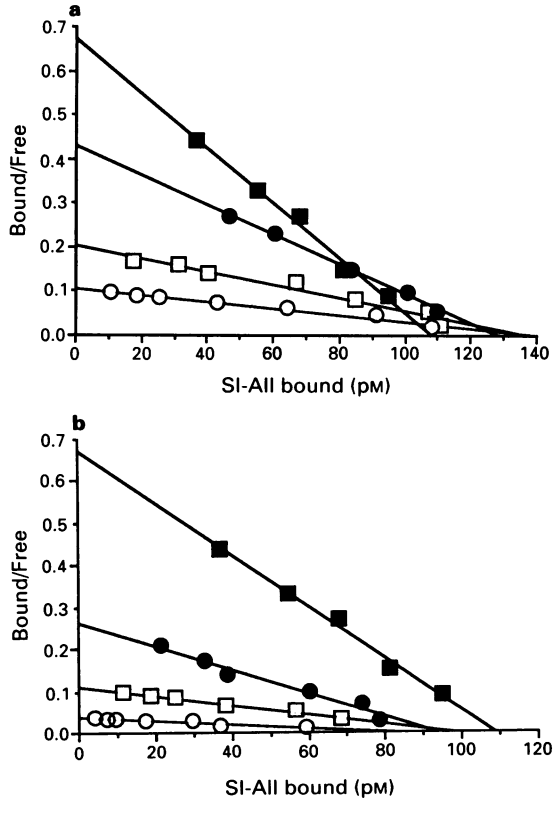


Figure 3 Scatchard plots of SI-AII binding to RASM cell membranes in the absence (■) or presence of 5 (●), 10 (□), or 50 (○) nM losartan (a) and 10 (●), 30 (□), or 100 (○) nM BMS-180560 (b). RASM membranes were pre-incubated for 15 min at 37°C with antagonists and incubated with radioligand [^{125}I]-SI-AII (0.1 nM) in the presence of increasing concentrations of SI-AII. Specific binding was determined using computer assisted LIGAND software program (Munson & Rodbard, 1980) and the data has been graphically displayed according to Scatchard. Results show representative data from experiments presented in Table 3.

Vascular smooth muscle contraction

The effects of losartan and BMS-180560 on AII-stimulated contractile responses in rabbit aortic rings are shown in Figure 4. Losartan (3 to 100 nM) produced parallel shifts to the right of the AII dose-response curve and did not affect maximum contractile responses (Figure 4a). The calculated K_B was 2.6 ± 0.13 nM. Data were also generated in the presence of 0.1% BSA in order to reproduce conditions used for the binding studies and phosphoinositide turnover measurements (see below). The Schild slope of these data was -0.9, and the calculated K_B was 6.7 ± 1.6 nM which was little different from that determined in the absence of BSA. BMS-180560 (0.1 to 1 nM) produced progressive shifts in the AII dose-response curve, and decreased the maximum AII-stimulated response by 25–75% (Figure 4c). Since AII was unable to overcome the inhibition by BMS-180560 the antagonism can be defined as insurmountable. The calculated apparent K_B was 0.068 ± 0.048 nM in the absence of BSA (Figure 4c) which was similar to the apparent K_B of 0.058 ± 0.016 nM calculated for EXP3174 (data not shown). By contrast, the K_B generated for BMS-180560 in the presence of BSA (5.2 ± 0.92 nM) was 76 fold greater (Figure 4d) and the insurmountable behaviour was maintained.

Figure 5 shows the specificity of BMS-180560 for AII receptors on rabbit aortic rings. At 1 nM, a concentration producing profound inhibition of AII-mediated contractions, BMS-180560 produced no effect on contractions elicited by KCl, noradrenaline, or the thromboxane A_2 receptor agonist, U-46619. The slight effect of BMS-180560 on endothelin-mediated contractions was attributed to differences in tissue sensitivity to ET. These data suggest BMS-180560 is a specific AII receptor antagonist.

Vascular smooth muscle cell activation determined with a Cytosensor microphysiometer

Exposure of RASM cells to AII produced a time and concentration-dependent increase in the extracellular acidification rates which reflects cellular activation (Owicki *et al.*, 1990). Since prolonged stimulation of cells (15 min) with AII desensitized the cells to subsequent stimulation, cumulative concentration-response curves were obtained with short application times. Stimulation of cells with AII for 10 s increased the extracellular acidification rates to a peak level (130% of basal rates after 2 min) which declined to basal rates during the next 12 min. This protocol provided reproducible responses to AII and allowed concentration-response curves to be obtained. Figure 6 shows the AII-stimulated increase in RASM extracellular acidification rates. The EC_{50} for AII was 18 ± 13 nM ($n = 10$) in control cells and in the presence of losartan (30 nM) the EC_{50} was increased 8 fold with no significant change in the maximum response, indicating simple competitive interaction. Assuming a Schild slope of unity (see above), the calculated K_B for losartan was 2.5 ± 1.0 nM, $n = 3$, which correlated with its potency as an inhibitor of rabbit aortic smooth muscle contraction ($K_B = 2.6$ nM). BMS-180560 at 0.01 nM had little effect on the EC_{50} for AII ($EC_{50} = 22$ nM) whereas 0.1 nM BMS-180560 increased the EC_{50} to 940 nM. The maximum responses to AII were decreased by 60% and 75% for both concentrations of BMS-180560 indicating insurmountable receptor antagonism. In these experiments BMS-180560 was more potent as an inhibitor of AII-stimulated cellular acidification than of contractile responses or phosphoinositide turnover (see below). These findings may relate to

the short AII stimulation protocol which was used for the cell based studies.

In order to compare the rates of dissociation of losartan and BMS-180560 from the RASM cells, cells were stimulated with AII in the absence of antagonist, and following exposure and washout of losartan and BMS-180560. Figure 7 shows the acidification rate of cells stimulated for 10 s initially, stimulated in the presence of losartan or BMS-180560, and stimulated after washout of drugs for 5 and 38 min. In the presence of losartan (50 nM) AII responses were decreased by 40% relative to control AII stimulation performed at the same time. BMS-180560 (0.1 and 1 nM) decreased AII responses by 22% and 100% respectively. Following washout of drugs for 5 min, AII responses to losartan had recovered to greater than control levels, probably reflecting protection of the AII receptors from desen-

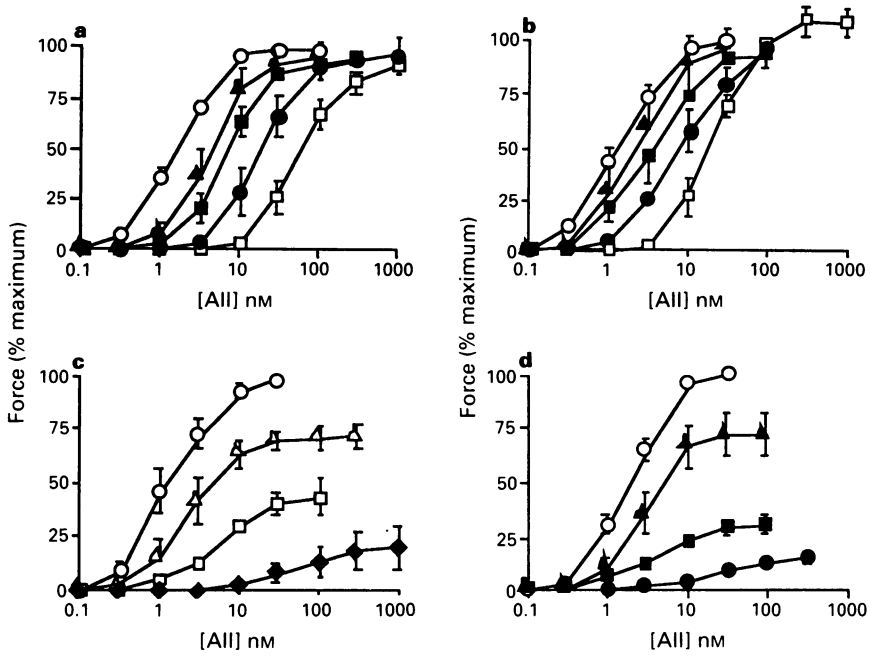


Figure 4 Effect of losartan and BMS-180560 on AII-stimulated contractile responses in rabbit aortic rings. Cumulative concentration-response curves elicited by AII were determined in the absence (○) or presence of: 3 (▲), 10 (■), 30 (●), or 100 (□) nM losartan (a,b). AII concentration-response curves were also obtained in the absence (○) or presence of 0.1 (△), 0.3 (□), 1 (◆), 3 (▲), 10 (■), or 30 (●) nM BMS-180560 (c,d). Experiments were conducted in the absence (a,c) or presence (b,d) of 0.1% BSA. Data shown are mean ± s.e.mean of at least 4 aortae from different rabbits.

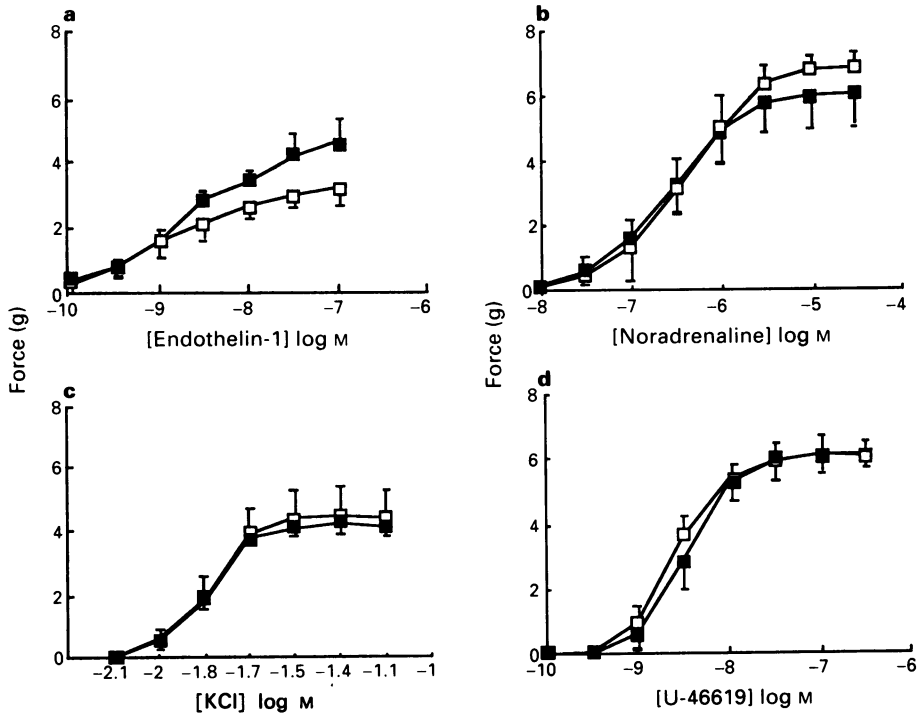
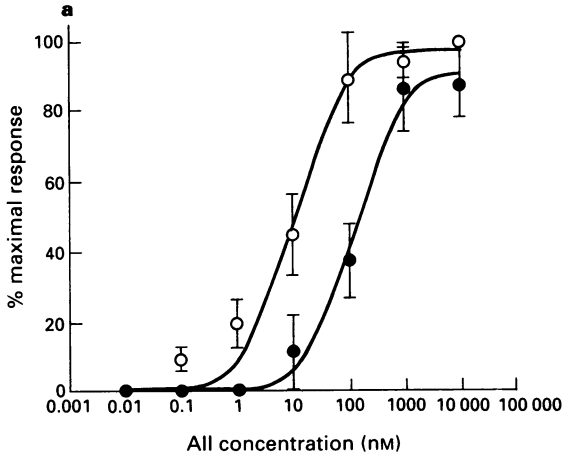


Figure 5 Effect of BMS-180560 on contractions elicited by endothelin-1 (a), noradrenaline (b), KCl (c), and U-46619 (d) in rabbit aortic rings. Aortic rings were exposed to the stated concentration of agonist in the absence (□) or presence (■) of 1 nM BMS-180560. Results are plotted as mean ± s.e.mean of 4 determinations.

sitization. Thus, AII-stimulated responses of control cells were decreased 40–45% during the course of this experiment. The inhibition of AII-stimulated responses by BMS-180560 (0.1 and 1 nM) were maintained following 5 min washout. More prolonged washing (38 min) was necessary in order to recover AII-stimulated responses following BMS-180560 treatment. This recovery was complete for cells exposed to 0.1 nM BMS-180560 and partial (70%) for cells treated with 1 nM BMS-180560. These findings indicate that losartan dissociates more rapidly from RASM cell AII receptors than the insurmountable antagonist BMS-180560 but both drugs can be considered reversible antagonists of AII-mediated responses.

Phosphoinositide metabolism

The ability of losartan and BMS-180560 to inhibit AII-stimulated phosphoinositide turnover was examined in RASM cells labelled to isotopic equilibrium with [^{3}H]-myoinositol. In the presence of LiCl (to inhibit inositol monophosphatase), AII (1 μM) produced a time-, and concentration-dependent increase in inositol mono-, bis-, and tris-phosphate which was linear for 0–40 min (data not shown). The IP_2/IP_3 fraction was 35–45% of the IP_1 fraction.



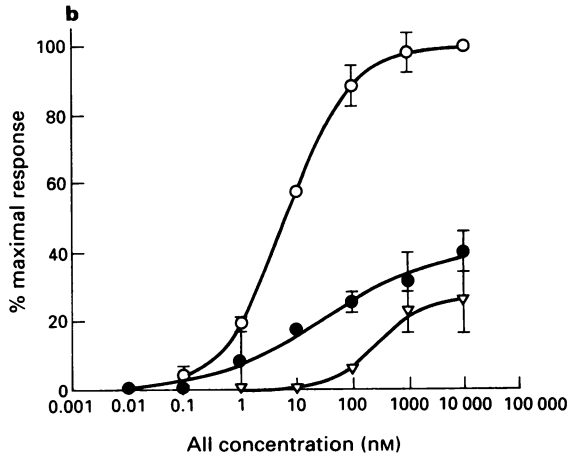


Figure 6 Effects of losartan and BMS-180560 on AII-stimulated increase in extracellular acidification rates of RASM cells. (a) RASM cells were preincubated for 20 min with media (○) or 30 nM losartan (●) and stimulated with the stated concentration of AII for 10 s. (b) Cells were preincubated with media (○), 0.01 nM (●), or 0.1 nM (▽) BMS-180560 followed by AII. Rates of extracellular acidification were measured with a Cytosensor microphysiometer. Peak increases in rates were computed, normalized to the maximum AII-stimulated increase in acidification rates, and plotted against the AII concentration. Results show mean ± s.e.mean of duplicate experiments which were performed three times.

The EC_{50} for AII-stimulated IP_1 response was 27 ± 6.9 nM ($n = 6$) and similar values were obtained for the increases in IP_2/IP_3 (data not shown). The maximum stimulation by AII varied somewhat with passage number, but routinely represented a 10 to 40 fold stimulation above basal IP_1 levels. The effect of losartan on AII-stimulated IP_1 formation in RASM cells is shown in Figure 8a. Losartan (30 and 200 nM) shifted the AII concentration-effect curve to the right and increased the EC_{50} for AII by 3 and 7 fold respectively. The effect of losartan was surmountable since higher concentrations of AII were able to overcome the inhibition by losartan, and the maximum AII-stimulated increase in IP_1 was similar in the absence or presence of antagonist. In marked contrast to the data obtained with losartan, its acid metabolite EXP3174 decreased the maximal AII-stimulated IP_1 formation in a concentration-dependent manner (Figure 8b). At a concentration of 1 nM EXP3174, and in the absence of BSA, maximal AII-stimulated responses were decreased by 50–60%. Thus, the insurmountable activity of EXP3174 (Wong & Timmermans, 1991) can be monitored at the level of second messanger formation.

The effects of BMS-180560 on AII-stimulated IP_1 formation is shown in Figure 8c. At a concentration of 3 nM and in the presence of 0.1% BSA, BMS-180560 produced somewhat variable effects on the AII concentration-response curve. Mean data demonstrated a small rightward shift of the curve and a 30% decrease in the maximum response. A higher concentration of BMS-180560 (10 nM) decreased the maximum IP_1 response by 80%. Losartan (100 nM) and BMS-180560 (10 nM) were used for these studies at equi-effective inhibitory concentrations such that at 10 nM AII, there was 85–90% inhibition of IP_1 formation. Increasing the AII concentration overcame the inhibition by losartan, but it was unable to overcome the inhibition by BMS-180560. Thus the inhibitory activity of BMS-180560 on IP_1 formation may be classified as insurmountable. The BMS-180560-induced decrease in maximal AII-stimulated IP_1 formation in RASM cells was quantitatively similar to that observed for AII-stimulated contraction of rabbit aortic smooth muscle (compare Figures 4d and 8c). Thus, in the presence of BSA, 3 and 10 nM BMS-180560 decreased maximum contractile force by 30 and 70% respectively, compared to 30 and 80% decreases in IP formation.

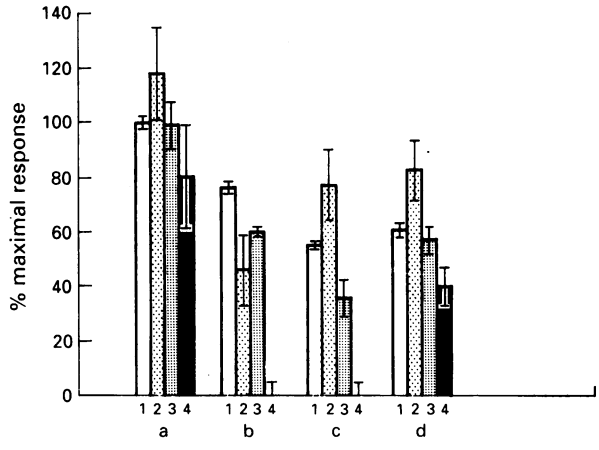


Figure 7 Reversal of losartan and BMS-180560 mediated inhibition of AII-stimulated RASM cell acidification. RASM cells were stimulated for 10 s with 100 nM AII in the absence (1) or presence of: 50 nM losartan (2), 0.1 nM BMS-180560 (3), or 1 nM BMS-180560 (4). (a) Initial responses to AII; (b) cells preincubated with drugs for 30 min and restimulated with AII; (c) drugs removed from the perfusate cells washed for 5 min and restimulated with AII; (d) cells washed for 38 min and restimulated with AII. Acidification rates were measured and normalized to rates obtained initially. Results are the mean ± s.e.mean of duplicate experiments which were performed 4 times.

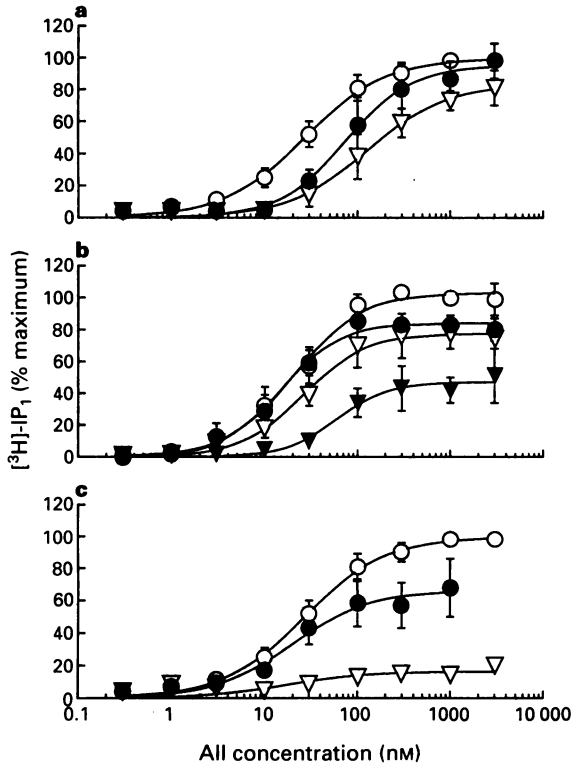


Figure 8 Effect of losartan, EXP3174, and BMS-180560 on AII-stimulated IP_1 formation in RASM cells. [^{3}H]-myoinositol-labelled RASM cell monolayers were incubated in the absence (○) or presence of 30 (●), and 200 (▽) nM losartan (a), or 0.1 (●), 0.3 (▽), or 1 (▼) nM EXP3174 (b) or 3 (●) and 10 (▽) nM BMS-180560 (c) for 15 min at 37°C, and stimulated for 30 min with increasing concentrations of AII. BSA (0.1%) was included in the experiments performed with losartan and BMS-180560. [^{3}H]-IP_1 was extracted as described in Methods and separated by anion exchange chromatography. IP_1 levels were normalized to % maximal stimulation produced by 1 μM AII. Results show mean curves ± s.e.mean of 4–6 determinations obtained with cells from different passage numbers.

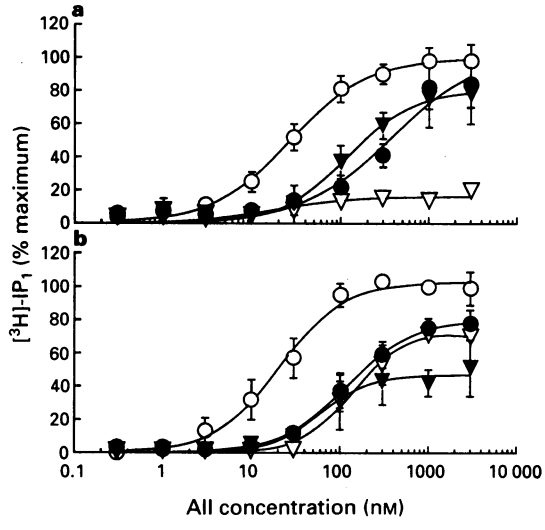


Figure 9 Reversal of BMS-180560 and EXP3174-mediated insurmountable inhibition of AII-stimulated IP_1 formation in RASM cells by losartan. [^{3}H]-myoinositol-labelled RASM cell monolayers were incubated in the absence (○) or presence of: 10 nM BMS-180560 (▽), 200 nM losartan (▼) and 200 nM losartan + 10 nM BMS-180560 (●) (a); or 200 nM losartan (●), 1 nM EXP3174 (▼), and 200 nM losartan + 1 nM EXP3174 (▽) (b) for 15 min at 37°C. For the combination experiment, cells were incubated with losartan for 15 min at 37°C, followed by BMS-180560 for 15 min. Cells were then stimulated with the stated concentrations of AII. [^{3}H]-IP_1 was extracted as described in Methods and separated by anion exchange chromatography. IP_1 levels were normalized to % maximal stimulation produced by 1 μM AII. Results show mean curves ± s.e.mean of 4–6 determinations obtained with cells from different passage numbers.

We have examined whether the inhibition by EXP3174 and BMS-180560 was irreversible by attempting to attenuate the antagonist-mediated inhibition by co-administration of losartan. Figure 9 shows that losartan was able to reverse the insurmountable inhibition by BMS-180560 and EXP3174. Thus, the maximum AII-stimulated IP_1 response was 20% of control in the presence of 10 nM BMS-180560, and 80% of control in the presence of 200 nM losartan (Figure 9a). The combination increased the AII-stimulated response to values not significantly different from those of losartan alone. Similarly, losartan reversed the EXP3174-mediated inhibition of AII-stimulated IP_1 formation to values not different from losartan alone.

Discussion

This study compared the interactions of the competitive AII receptor antagonist, losartan and the insurmountable antagonists, BMS-180560 and EXP3174 with the AT_1 receptor. Losartan and BMS-180560 were potent inhibitors of [^{125}I]-SI-AII binding to rat adrenal cortex and vascular AT_1 receptors with K_i values of 9 and 16 nM for losartan, and 18 and 7 nM for BMS-180560 respectively. The value obtained for BMS-180560 was dependent on the concentration of BSA in the assay buffer since lowering the BSA concentration to 0.01% decreased the K_i by 23 fold. By contrast, the K_i of losartan was little affected by lowering the BSA concentration whereas its structurally related acid analogue EXP3174, exhibited an 8 fold decrease in K_i in low (0.07%) BSA. These findings were quantitatively similar to those reported by Chiu *et al.* (1991). This property probably relates to the physicochemical characteristics of these molecules. Thus, it has been suggested that the presence of a di-acidic function in the AII receptor antagonist molecule may be responsible for protein binding. Chiu *et al.* (1991) demonstrated that 99% of the diacid DuP 532 bound to BSA, compared to 60% for losartan, which contains a mono-acidic function. We have also examined the protein binding activities of a series of mono and diacid AII antagonists and the protein binding activity was related to, but was not totally dependent upon, the presence of a diacid function (data not shown). From the possible binding sites on BSA (Kragh-Hansen, 1981) it is likely that AII receptor antagonists bind at the bilirubin site 3, or the common drug binding site 6. Our results with BMS-180560 indicate that a substantial amount of drug (>90%) was bound to BSA, thereby reducing the concentration of free drug able to interact with the AT_1 receptor. Thus, BMS-180560 exhibited a K_B for rabbit aortic smooth muscle AII receptors of 0.068 nM in the absence and 5.2 nM in the presence of 0.1% BSA, indicating >95% binding of drug to BSA. The recently described Glaxo antagonist, GR 117,289, is a diacid which also shows significant binding to BSA (Robertson *et al.*, 1992). The pronounced binding of these drugs to plasma and tissue proteins may provide an explanation, in part, for the long duration of action of these diacid AII receptor antagonists.

Saturation binding experiments conducted in the presence of losartan indicated that the interaction of losartan with the vascular AT_1 receptor was competitive. Thus, in the presence of losartan, the K_D of SI-AII was increased to higher values, with no change in the B_{max}. Moreover, Schild slopes of the saturation binding data were close to unity suggesting a competitive interaction of losartan with the AT_1 receptor.

The interaction of BMS-180560 with the AT_1 receptor appeared competitive at low concentrations of BMS-180560, since the K_D for SI-AII was increased with no change in B_{max} value. Similar results have been reported for the insurmountable antagonists L-159,809 and CV-11974. Thus, L-158,809 and CV-11974 at concentrations similar to their K_i values increased the K_D of [^{125}I]-SI-AII but had no effect on B_{max} (Chang *et al.*, 1992; Shibouta *et al.*, 1993). However, at high concentrations of BMS-180560 (10 to 30 fold greater than its K_i for vascular AII receptors) the K_D for SI-AII was increased 10 to 20 fold and the B_{max} value was decreased by 12–45%. These findings suggest that BMS-180560 may exhibit mixed (competitive and non-competitive) inhibitory activity at high concentrations. The structurally similar insurmountable antagonist, GR 117,289 has also been reported to produce both decreases in B_{max} and increases in K_D for [^{3}H]-AII binding to rat liver membranes (Robertson *et al.*, 1992). Similar results were also reported for EXP3174 which decreased the B_{max} and increased the K_D for [^{125}I]-AII binding to rat lung membranes (Wienen *et al.*, 1992). However in contrast to our findings, inhibition was obtained at concentrations of EXP3174 which were close to its K_i value for the AII receptor. These discrepancies could result from differences in the AT_1 receptor subtypes in liver compared to the smooth muscle (Widdowson *et al.*, 1993) although both liver and lung are reported to contain predominantly the AT_{1a} receptor subtype (Kakar *et al.*, 1992; Widdowson *et al.*, 1993). Alternatively, slow kinetics of antagonist binding may have contributed to the degree of inhibition obtained. Thus, Pendleton *et al.* (1989) showed that the insurmountable peptide antagonist, Sar^{1}Leu^{8}AII reduced the B_{max} of [^{125}I]-AII binding sites on rabbit adrenal membranes only if membranes were preincubated with antagonist, whereas coincubation resulted in competitive inhibition. Decreases in B_{max} values were therefore attributed to slow dissociation of the peptide antagonist from the receptor. In our study the radioligand reached equilibrium during the course of the 2 h incubation in the absence or presence of BMS-180560 (3–30 nM) and preincubation of membranes with BMS-180560 did not change the competitive nature of the inhibition. These data suggest that BMS-180560 had reached equilibrium with the vascular AII receptor and at concentrations similar to its K_i value, BMS-180560 functioned as a competitive inhibitor. Concentrations of BMS-180560 which decreased B_{max} values (100–300 nM) were greatly in excess of those required to depress AII-stimulated contraction of rabbit aorta (3–30 nM) when measured in the presence of BSA. Liu *et al.* (1992) proposed that insurmountable antagonism of a series of AII receptor analogues was related to peptide-dependent internalization of AII receptors thereby decreasing receptor concentration. However, our data do not support the concept that BMS-180560 decreased receptor number at concentrations of BMS-180560 which produced insurmountable antagonism.

Losartan functioned as a classical competitive antagonist of AII-mediated smooth muscle contraction. Thus, AII concentration-response curves were shifted to the right with no depression of maximum response. These findings confirm reports of others who demonstrated losartan was a competitive inhibitor of AII-stimulated rabbit aortic contraction (Wong *et al.*, 1990a,c; Liu *et al.*, 1992; Wienen *et al.*, 1992; Robertson *et al.*, 1992; Cazaubon *et al.*, 1993; Shibouta *et al.*, 1993). In contrast BMS-180560 decreased the maximum AII-stimulated contraction of rabbit aorta in a concentration dependent manner and at 1 nM BMS-180560 (in the absence of BSA) maximum contractile responses were inhibited by 75%. Similar results have been reported for L-158,809 (Chang *et al.*, 1992), GR 117,289 (Robertson *et al.*, 1992) and EXP3174 (Wong & Timmermans, 1991; Wienen *et al.*, 1992), although the maximum reduction observed for EXP3174 was only 24% (Wienen *et al.*, 1992). Liu *et al.* (1992) demonstrated concentration-dependent saturable inhibition of maximum AII-stimulated contraction of rabbit aorta by a series of peptide AII analogues and the depression of AII contraction maxima correlated with the pA_2 values of the antagonists for the AII receptor. However, in the case of the nonpeptide antagonists, the ability of these drugs to decrease AII contraction maxima appears not to correlate with potency. Thus, BMS-180560 produced substantially greater decreases than EXP3174 although the antagonists had similar apparent K_B and K_i values for AII receptors.

This study also examined the effects of AII antagonists on smooth muscle cells using extracellular acidification as a monitor of cellular activation. AII stimulated the acidification rates of smooth muscle cells in a concentration-dependent manner and with an EC_{50} of 18 nM which correlated with the EC_{50} for AII-stimulated phosphoinositide turnover (27 nM) suggesting that phospholipase C (PLC) stimulation may be correlated with cellular activation. Losartan shifted the AII concentration-response curve in a manner commensurate with competitive inhibition and with a potency which was similar to its interaction with AII receptors on rabbit aortic rings. By contrast, BMS-180560 produced insurmountable inhibition of AII-stimulated cellular activation with increases in EC_{50} values and depression of AII maximal responses. The magnitude of this inhibition was similar to that observed for inhibition of AII-stimulated contraction of rabbit aortic rings and phosphoinositide turnover although these effects were observed at lower concentrations of BMS-180560. Robertson *et al.* (1992) reported differences in the potency of GR 117,289 in functional studies compared to its affinity for AII binding sites; these parameters may be influenced by pretreatment time, protein binding, drug lipophilicity, or receptor concentration. It is also possible that the short stimulation protocol used for the microphysiometer experiments did not allow AII to compete effectively with AII receptors which were occupied with BMS-180560 especially if BMS-180560 dissociated slowly. Indeed, washout studies indicated that BMS-180560 did dissociate from the RASM cells slower than losartan but that functional responses to AII could be restored following more extensive washing. These observations indicate that BMS-180560 binding to the RASM cell AII receptor was reversible.

AT_1 receptors on RASM cells transduce their signal in part by activation of a PLC (Smith *et al.*, 1984; Griendling *et al.*, 1987; 1991). Phosphoinositide turnover is enhanced with production of diacylglycerol, and inositol trisphosphate. The elevation of intracellular Ca^{2+} is thought to be ultimately responsible for the increased contractile state of smooth muscle. In order to define the site at which BMS-180560 exerted its insurmountable action, IP_1 formation was determined as a measure of PLC activation. Losartan shifted the AII-mediated IP_1 formation to the right with no change in the maximal response. Thus, losartan behaved as a competitive antagonist of AII-mediated responses whether the determinations were made at the level of receptor binding, IP_1 formation, smooth muscle contraction, or cellular activation (measured as extracellular acidification). Others have reported the ability of losartan to inhibit AII-mediated second messenger formation such as Ca^{2+} transients (Chiu *et al.*, 1990), phosphoinositide turnover (Pfeilschifter, 1990), aldosterone secretion (Balla *et al.*, 1991) but demonstration of the competitive nature of this inhibition has been lacking. By contrast BMS-180560 functioned as an insurmountable antagonist of AII-mediated vascular contractile responses, extracellular acidification and IP_1 formation. Both BMS-180560 and EXP3174 decreased maximal AII-stimulated IP_1 formation in an insurmountable manner. Chang *et al.* (1992) have also reported that L-158,809 shifted the AII-stimulated [^{3}H]-IP accumulation in RASM cells to the right and significantly decreased the maximal response. Inhibition of AII-stimulated phosphoinositide turnover by BMS-180560 was quantitatively similar to that for AII-stimulated contraction of rabbit vascular smooth muscle, when measurements were made under the same conditions of BSA concentration.

Our IP measurements were made after 30 min of cell stimulation. Wojcikiewicz et al. (1993) have recently reviewed the problems of measuring IP formation in Li-containing assays after long time intervals (>5 min) of cell stimulation. Moreover, our measured [^{3}H]-IP_1 may have derived from both PIP_2 and PI hydrolysis. Griendling et al. (1991) have described two isoforms of PLC in RASM cells with different Ca^{2+}, pH and substrate specificities. Thus, it was possible that the measured [^{3}H]-IP_1 pool derived from activation of two PLCs. Since IP_2 and IP_3 are thought to be generated in RASM cells as a result of rapid PLC-mediated PIP_2 hydrolysis (Griendling et al., 1991), it was important to establish that the insurmountable activity was also demonstrable at short time intervals using IP_3 formation as a monitor of PIP_2 hydrolysis. We have demonstrated that EXP3174 and BMS-180560 (at 1 nM) produced insurmountable antagonism of AII-stimulated IP_2/IP_3 formation at 4 min (data not shown). Since the insurmountable activity was detected at this level, it follows that the insurmountable target site for these antagonists lies proximal to PLC-mediated PIP_2 hydrolysis.

The interaction of insurmountable antagonist and receptor is not irreversible since losartan reversed the insurmountable inhibition of AII-stimulated phosphoinositide turnover caused by EXP3174 and BMS-180560 and inhibition of AII-stimulated increase in RASM extracellular acidification rates by BMS-180560 was irreversible following washout. These data confirm previous reports showing losartan was able to attenuate EXP3174-induced decrease in maximum AII contraction of vascular smooth muscle (Wong & Timmermans, 1991). Moreover, these findings suggest that the reversible antagonist losartan but not AII was able to access the BMS-180560 binding site which was responsible for insurmountable inhibition.

The molecular basis for insurmountable inhibition remains to be established although a number of theories have been proposed. Receptor heterogeneity could explain this phenomenon although available evidence suggest that the AII receptors on RASM cells represent a homogeneous population (Kakar et al., 1992; Cohen et al., 1993). Pseudoirreversible antagonism and allosteric modulation of AII receptors have also been proposed as the basis for insurmountable antagonism although our studies were unable to discriminate between these alternatives. Pseudoirreversible antagonism, which decreases the available receptors for effector coupling, could be produced by slow dissociation of the antagonist from the receptor. Our studies indicated that BMS-180560 dissociated slower than losartan and slow dissociation rates have also been reported for EXP3174 (Chiu et al., 1991). Thus, replacement of receptor-bound BMS-180560 or EXP-3174 by the rapidly dissociating antagonist losartan would allow AII to compete more effectively. This mechanism could explain the reversibility by losartan of BMS-180560- or EXP3174-induced insurmountable inhibition.

An allosteric model has also been proposed to explain insurmountable antagonism at AII receptors (Timmermans et al., 1991). Two binding sites are postulated, one which binds AII BMS-180560, and losartan and a second site which could allosterically decrease receptor-effector coupling thereby producing depression of the maximal AII-stimulated responses. The first site is demonstrable with AII radioligands and AII and nonpeptides compete for binding to this site. The second site is not available to AII whereas BMS-180560, EXP3174, and losartan can all bind. However, only BMS-180560 and EXP3174 can allosterically decrease receptor-effector coupling and cause insurmountable inhibition, perhaps by stabilizing a receptor conformation which had reduced ability to couple to G proteins. Binding of losartan (but not AII) to this site would be predicted to reverse BMS-180560- or EXP3174-mediated insurmountable inhibition. These two sites may have similar molecular characteristics since insurmountable and surmountable antagonists differ only slightly in their structure. Thus, insurmountable activity is associated with the presence of a -COOH group whereas the parent $-CH_2OH$ containing drug generally exhibits surmountable antagonism. Perhaps more convincing are data for a series of imidazol-2-one AII receptor antagonists, where modest changes in alkyl substitution changed the nature of the antagonism (Reitz et al., 1993). Thus, methyl and isopropyl containing drugs were surmountable whereas ethyl substitution resulted in an insurmountable antagonist. Whether this behaviour is due to differential dissociation rates of the compounds or their abilities to bind to secondary allosteric sites remains to be established.

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