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Angiotensin II receptor subtypes and contractile responses in portal vein smooth muscle

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Abstract

The selective biphenylimidazole and tetrahydroimidazopyridine antagonists exemplified by losartan (DuP 753) and PD 123319 have been shown to bind selectively to angiotensin AT_1 and AT_2 receptor subtypes, respectively. To characterize which subtypes of angiotensin II receptors are expressed in mammalian portal vein smooth muscle, we performed, using both membrane and strip preparations, [^{3}H]angiotensin II binding experiments and then contraction experiments to investigate the functional relevance of these binding sites. Specific binding of [^{3}H]angiotensin II was of high affinity, saturable and reversible. Specific binding of [^{3}H]angiotensin II was completely displaced by angiotensin II and the peptide antagonist [Sar^1,Ile^8]angiotensin II. The inhibition of [^{3}H]angiotensin II binding by losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole, potassium salt) and DuP 532 (2-*n*-propyl-4-pentafluoroethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole-5-carboxylic acid) was biphasic and LIGAND curve-fitting analysis revealed two populations of specific binding sites. One subpopulation represented 75% of the total binding and showed high affinity for angiotensin II, losartan and DuP 532, but low affinity for the peptide angiotensin AT_2 receptor antagonist CGP 42112A (*N*-α-nicotinoyl-Tyr-Lys-[*N*-α-CBZ-Arg]-His-Pro-Ile-OH) and thus appeared identical to the cloned angiotensin AT_1 receptor subtype. The remaining 25% of the sites showed nearly 1000-fold lower affinity for losartan, 6500-fold lower affinity for DuP 532 and high affinity for PD 123319 (*S*-1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-diphenylacetyl-4,5,6,7-tetrahydro-1*H*-imidazo-[4,5-*c*] pyridine-6-carboxylic acid, difluoroacetate monohydrate) and CGP 42112A, with values of K_i in the same range (nM) as those found for losartan and DuP 532 at angiotensin AT_1 binding sites. These sites appear to be angiotensin AT_2 receptors. Only the angiotensin AT_1 receptor subtype interacted with G-proteins, as indicated by the 80% inhibition of [^{3}H]angiotensin II binding in the presence of guanosine 5′-O-(3-thiophosphate) or fluoroaluminates. Although the angiotensin II-induced contraction was completely inhibited by losartan with a pA_2 value of 8.8, PD 123319 reduced the angiotensin II-induced contraction by 20–25%, indicating that both angiotensin AT_1 and AT_2 receptor subtypes are functional in portal vein smooth muscle.

Keywords: Angiotensin II; Losartan; DuP 532; PD 123319; CGP 42112A; Smooth muscle, portal vein

1. Introduction

Isolated portal vein preparations from rats are known to display spontaneous myogenic activity which is sensitive to physiologically relevant concentrations of angiotensin II (Bohr and Uchida, 1967; Blair-West et al., 1971; Hamon and Worcel, 1982). The stimulating effect of angiotensin II has been demonstrated to be mediated by angiotensin receptors (Scanlon and Moore, 1988). Two pharmacologically distinct subtypes of angiotensin II receptors (AT_1 and AT_2) have been defined by radioligand-receptor binding studies using synthetic antagonists (Wong et al., 1992; Bottari et al., 1993; Timmermans et al., 1993). The non-peptide biphenylimidazole antagonists exemplified by losartan and DuP 532 have a high affinity for angiotensin AT_1 receptors but a low affinity for angiotensin AT_2 receptors. Conversely, the non-peptide tetrahydroimidazopyridines such as PD 123177 or PD 123319 and the peptidergic antagonist, CGP 42112A, both have a high affinity for angiotensin AT_2 receptors and a lower

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affinity for angiotensin AT_1 receptors (Bottari et al., 1991; Whitebread et al., 1991).

The two subtypes can also be separated on the basis of their susceptibility to reducing agents, like dithiotreitol, since radiolabelled angiotensin II binding to angiotensin AT_1 receptors is reduced with dithiotreitol whereas binding to angiotensin AT_2 receptors is either enhanced or unaffected (Chang and Lotti, 1990; Rogg et al., 1990). Furthermore, their ability to respond to angiotensin II and angiotensin III and the second messenger systems to which they are coupled is different. Whereas angiotensin AT_1 receptors are coupled to ion channels and phosphoinositide breakdown and appear to mediate most of the known effects of angiotensin II, the coupling of angiotensin AT_2 receptors is largely unknown. However, recent studies have shown that, in some cell types, angiotensin AT_2 receptors can modulate guanylate cyclase activity (Sumners et al., 1991; Bottari et al., 1992) and T-type Ca^{2+} channels (Buisson et al., 1992). However, the function of angiotensin AT_2 receptors has not been completely elucidated, particularly in the cardiovascular system.

Recently, it was proposed that angiotensin AT_1 and AT_2 receptors can be further subdivided into AT_{1a}, AT_{1b}, AT_{2a} and AT_{2b} types based on their pharmacological profiles and the results of molecular biological experiments (Ernsberger et al., 1992; Iwai and Inagami, 1992; Tsutsumi et al., 1992; Tsutsumi and Saavedra, 1992; Widdowson et al., 1993).

In the present study, we used a combination of ligand binding studies and contraction experiments to identify the angiotensin II receptor subtypes in portal vein smooth muscle and their respective roles in the physiological contractile response. The results obtained show that the angiotensin II-induced contraction is mediated through activation of both angiotensin AT_1 and AT_2 receptor subtypes. The angiotensin AT_1 receptor subtype which interacts with G-proteins is responsible for about 80% of the maximal contractile response.

2. Materials and methods

2.1. Membrane preparation and binding assay

Equine portal veins (Abattoir of Bordeaux) were removed by the veterinary surgeon and put in a ice-cold container before being carried (15 min) to the laboratory. About 70 g of fresh material was minced with scissors and homogenized with a polytron in 40 mM Hepes-NaOH (4° C, pH 7.4) containing 0.3 M sucrose and protease inhibitors (1 mM iodoacetamide and 0.1 mM phenylethylsulfonyl fluoride). The homogenate was centrifuged at $1000 \times g$ for 10 min and at $10000 \times g$ for 20 min (4° C) to remove cellular debris. The supernatant was stirred with 0.5 M KCl for 20 min (4° C) to solubilize contractile proteins and centrifuged again at $100000 \times g$ for 20 min (4° C). The resulting pellet was suspended in fresh ice-cold 20 mM Hepes-NaOH buffer (pH 7.4) and homogenized with a Teflon pestle and a glass homogenizer at 1200 rpm. Protein concentration was determined according to Bradford (1976). The protein suspension (5–6 $mg \cdot ml^{-1}$) was divided into 1 ml aliquots and then frozen and stored at −80° C until used.

Assays were conducted in a total volume of 2 ml in polyethylene tubes (Polylabo, France). Each tube contained 100 μl membrane suspension, various concentrations of [^{3}H]angiotensin II over the range of 0.1 to 6 nM (for equilibrium studies), 20 mM Hepes buffer (pH 7.4) containing 0.1% bovine serum albumin (w/v) and a protease inhibitor (bacitracin, 0.05 $mg.ml^{-1}$). Incubations were initiated by the addition of membrane and were carried out for 60 min at 25° C. Non-specific binding was defined in the presence of 2 μM unlabelled angiotensin II and was subtracted from the total binding. After incubation, bound and free ligand were separated by vacuum filtration, using a cell harvester (Millipore), over Whatman GF/C glass-fiber filters which were pre-soaked in 0.1 M Tris-HCl for 1 h at 4° C. The filters were washed 3 times with 8 ml ice-cold 0.1 M Tris-HCl, and the radioactivity was counted by liquid scintillation at 55% efficiency in a Packard Tri-Carb 1500 counter.

2.2. Strip preparation and binding assay

Male Wistar rats (160–300 g) were killed by cervical dislocation. Five animals were generally used for each experiment. The portal vein was immediately removed and incubated, before dissection, in a physiological solution containing (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 0.24, Hepes 8, glucose 11 (pH 7.4, 37° C). Fat and connective tissue were rapidly removed and the portal vein was cut in six to eight longitudinal strips (0.5–1.0 mg wet weight; Morel and Godfraind, 1987; Dacquet et al., 1989).

Assays were conducted in a total volume of 1 ml in polyethylene 24-well plates (Polylabo, France). Each well contained one to two strips, various concentrations of [^{3}H]angiotensin II over the range of 0.1–10 nM (for equilibrium studies) and the physiological solution (pH 7.4, 37° C) containing a protease inhibitor (bacitracin 0.05 $mg \cdot ml^{-1}$). At the end of the incubation period, each strip was dried on filter paper and then weighed. Radioactivity was measured by dissolving the vein strips in 100 μl NaOH 0.1 M. Non-specific binding was defined as the amount of radioligand bound in the presence of 2 μM unlabelled angiotensin II and was subtracted from the total binding.

2.3. Contraction experiments

Isometric contractions of longitudinal strips from rat portal vein were recorded in an experimental chamber described previously (Mironneau et al., 1980) by means of a highly sensitive isometric force transducer (Akers 801 AME, Norten, Norway). The physiological solution was similar to that used for binding experiments with intact strips. The circulating solution was maintained at $37 \pm 1°$ C. Vein strips were first contracted with angiotensin II (10 nM, maximal response) and then washed for 30 min. Subsequent contractions in response to angiotensin II in the presence of various antagonists were expressed as a percentage of this maximal contraction to angiotensin II.

2.4. Protease inhibitors

All the dilutions of [^{3}H]angiotensin II, angiotensin II and [Sar^1,Ile^8]angiotensin II were made with buffer containing 0.05 mg · ml^{-1} bacitracin. The binding buffer also contained 0.05 mg.ml^{-1} bacitracin, which was effective in inhibiting peptide break down but did not interfere with radioligand binding.

2.5. Chemicals

5-L-Isoleucine [tyrosyl-3,5-^{3}H]angiotensin II (specific activity 51 and 38 Ci · mmol^{-1}) was obtained from Amersham (Les Ulis, France). Angiotensin II, [Sar^1,Ile^8]angiotensin II, bacitracin and bovine serum albumin were from Sigma (St Quentin-Fallavier, France). DuP 753 (Losartan) (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole, potassium salt) and DuP 532 (2-*n*-propyl-4-pentafluoroethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole-5-carboxylic acid) were from Dupont Merck (USA) and PD 123319 (*S*-1-[[4-dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo-[4,5-*c*]pyridine-6-carboxylic acid, difluoroacetate mono hydrate) was from Parke Davis (USA). CGP 42112A (*N*-α-nicotinoyl-Tyr-Lys[N-α-CBZ-Arg]-His-Pro-Ile-OH) was from Neosystem Laboratories (Strasbourg, France). Other compounds were obtained from Sigma (St Quentin-Fallavier, France) and Merck (Nogent-sur-Marne, France).

2.6. Statistical analysis

The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]angiotensin II were estimated by Scatchard analysis of the saturation data. The ability of unlabelled agonists and antagonists to inhibit [^{3}H]angiotensin II binding was estimated from the IC_{50} value, which was the concentration which inhibited 50% of the maximal response. A value for the inhibition constant K_i was calculated from the equation, $K_i = IC_{50}/(1 + L/K_d)$, where L was the concentration of [^{3}H]angiotensin II (Cheng and Prusoff, 1973). The Hill coefficient for inhibition of a drug was obtained by Hill plot analysis. The data obtained from saturation and competition studies were analyzed by a program derived from the non-linear least-squares curve-fitting program LIGAND (Munson and Rodbard, 1980). The data were first fitted to a one- then a two-site model and if the residual sums of squares were statistically less for a two-site fit of the data than for a one-site, as determined by an F-test comparison, the two-site model was accepted. In these data, n represents the number of experiments, each done in duplicate. The experimental results are expressed as means ± S.E.M. and significance was tested by Student's t-test. P values smaller than 0.05 were considered as significant.

3. Results

3.1. Characteristics of [^{3}H]angiotensin II binding in intact strips and membranes of portal vein smooth muscle

Insets of Fig. 1 illustrate the equilibrium binding of [^{3}H]angiotensin II to intact strips and membranes of portal vein smooth muscle. In each case, specific binding was a hyperbolic function of the radioligand concentration and was saturable. Scatchard analysis of these data resulted in a straight line, suggesting the presence of a single class of binding sites with a K_d of 2.1 ± 0.3 nM and a B_{max} of 17.7 ± 1.6 fmol/mg wet weight ($n = 6$ for strips, Fig. 1A). The specific binding represented 80% of the total amount of bound ligand at the K_d value. Binding of [^{3}H]angiotensin II to portal vein membranes (Fig. 1B) was of similar affinity ($K_d = 1.7 \pm 0.7$ nM) with a binding capacity of 30.1 ± 3.3 fmol/mg of protein ($n = 6$).

Specific binding of [^{3}H]angiotensin II in intact strips was also determined as a function of the incubation time in physiological solution. Association reached a plateau after 40–50 min incubation at 37° C (Fig. 2A). The inset shows that the semi-logarithmic representation of the data was linear, as expected for a pseudo-first-order reaction (Weiland and Molinoff, 1981):

$$\ln[LRe]/([LRe] - [LR]) = k_1 t.[L_T][R_T]/[LRe]$$

where $[L_T]$ is the total concentration of [^{3}H]angiotensin II, $[R_T]$ is the total concentration of specific binding sites, [LRe] is the concentration of [^{3}H]angiotensin II-receptor complex at equilibrium and [LR] is the concentration of the complex at time t. The slope of this plot, K_{obs}, was estimated in six different experiments to be 0.042 ± 0.010 min^{-1}. Furthermore, K_{obs} =

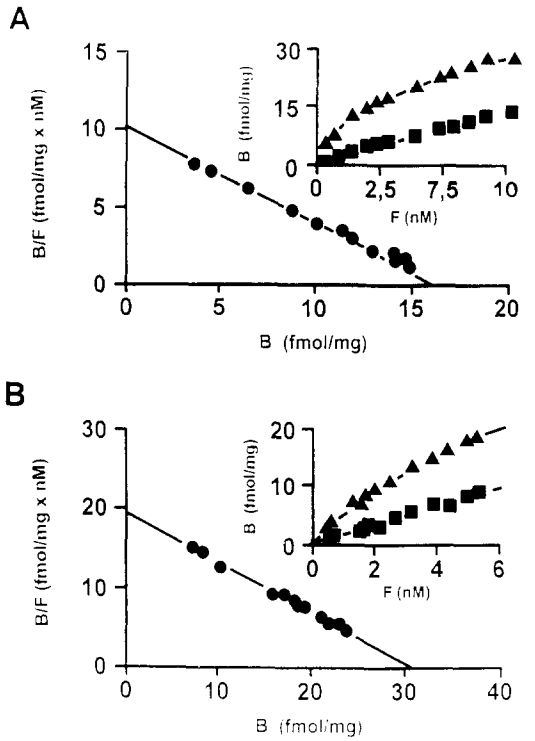

Fig. 1. Specific binding of [^{3}H]angiotensin II to intact strips and membranes from portal vein smooth muscle. (A) Inset, saturation binding experiments were carried out with portal vein strips (0.5–1.0 mg wet weight) incubated with increasing concentrations of [^{3}H]angiotensin II (0.1–10 nM) for 60 min at 37° C. Each point is the mean ± S.E.M. of six experiments, each carried out in duplicate. (B) Inset, saturation binding experiments were carried out with portal vein membranes (0.15 to 0.25 mg.ml^{-1} of protein) incubated with 0.1–6 nM [^{3}H]angiotensin II for 60 min at 25° C. Each point is the mean ± S.E.M. of six experiments, each carried out in duplicate. In each case, non-specific binding (defined with 2 μM unlabelled angiotensin II) represented about 15% of total binding at 1 nM [^{3}H]angiotensin II. Total binding (▲), non-specific binding (■), specific binding (●). Scatchard analysis of specific binding was carried out using the non-linear least-squares curve fitting program LIGAND (Munson and Rodbard, 1980). B/F, bound/free.

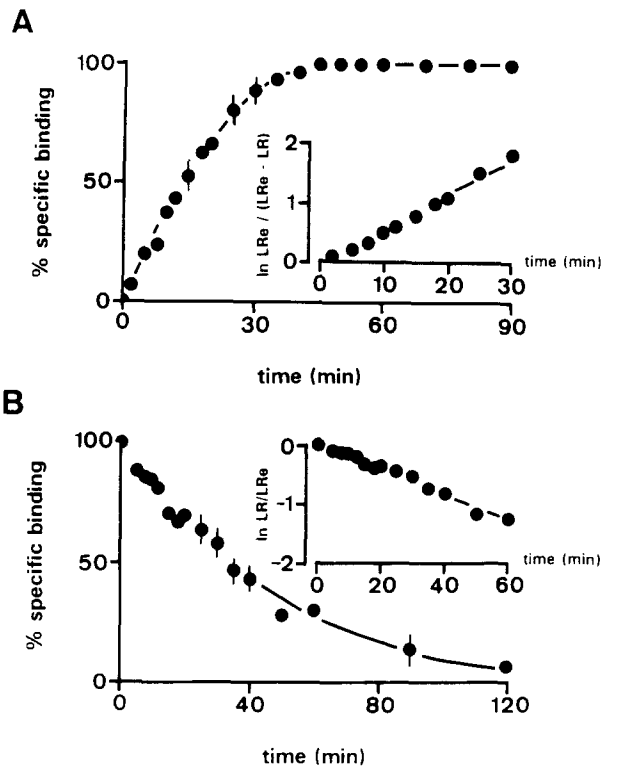

Fig. 2. Association and dissociation kinetics of [^{3}H]angiotensin II in rat portal vein strips. (A) Association was initiated by adding 1 nM [^{3}H]angiotensin II to intact vein strips bathed in a physiological solution. Specific binding was determined between 0 and 90 min. Inset, semilogarhithmic plot of association data. (B) Dissociation was initiated after equilibrium had been reached, by adding 2 μM unlabelled angiotensin II. Inset, semilogarhithmic plot of dissociation data. Non-specific binding defined in the presence of 2 μM unlabelled angiotensin II was constant and represented 15% of total binding. LRe, concentration of [^{3}H]angiotensin II-receptor complex at equilibrium; LR, concentration of the complex at time t. Each value is the mean ± S.E.M. of six experiments, each carried out in duplicate.

$k_1[L_T] + k_{-1}$, in which k_1 and k_{-1} represent the rate constants of association and dissociation of the [^{3}H]angiotensin II-receptor complex, respectively. The binding of labelled angiotensin II to intact vascular strips was a reversible process. After equilibrium was reached, dissociation of the complex was initiated by adding a large excess (2 μM) of unlabelled angiotensin II (Fig. 2B). The dissociation was a first-order reaction and the rate constant k_{-1} was calculated to be 0.019 ± 0.010 min^{-1} ($n = 6$). The calculated value of k_1 was 0.023 ± 0.010 min^{-1}.nM^{-1} ($n = 6$) and the dissociation constant from kinetic data ($K_d = k_{-1}/k_1$) was estimated to be 0.83 ± 0.20 nM, which was close to that, 2.1 ± 0.3 nM, determined from equilibrium binding experiments. Similar kinetic data were obtained for

Table 1
Inhibition of 1 nM [^{3}H]angiotensin II binding to portal vein strips and isolated membranes by angiotensin II and a peptide antagonist, [Sar^1,Ile^8]angiotensin II.

Antagonists	Intact strips		Isolated membranes	
	K_i (nM)	n Hill	K_i (nM)	n Hill
Angiotensin II	0.97 ± 0.05	1.01 ± 0.04	0.72 ± 0.20	1.02 ± 0.05
[Sar^1,Ile^8]angiotensin II	0.34 ± 0.02	1.03 ± 0.05	0.20 ± 0.01	1.07 ± 0.07

Non-specific binding was approximately 15% of total binding. Each value is the mean ± S.E.M. of three to four experiments, each carried out in duplicate.

vascular membranes with K_{obs} and k_{-1} values of 0.06 ± 0.01 min^{-1} and 0.018 ± 0.010 min^{-1}, respectively ($n = 5$). The ratio k_{-1}/k_{+1} gave a K_d value of 0.43 ± 0.06 nM, which was close to that obtained from equilibrium binding experiments (1.7 ± 0.7 nM).

3.2. Inhibition of [^{3}H]angiotensin II binding to intact strips and membranes of portal vein smooth muscle

In both intact strips and isolated membranes, unlabelled angiotensin II and [Sar^1,Ile^8]angiotensin II inhibited the [^{3}H]angiotensin II specific binding in a monophasic manner. The inhibition constant (K_i) and the Hill coefficient are listed in Table 1. These peptides showed nearly identical potencies on both intact strips and membranes, indicating that disruption of the cells did not change markedly the properties of the angiotensin II receptors in mammalian portal vein smooth muscles. In contrast, two non-peptide antagonists of the angiotensin AT_1 receptors, DuP 532 and losartan, displaced [^{3}H]angiotensin II binding in a biphasic manner (Fig. 3A). The curves were best fitted with a two-site model, which is expressive of an interaction with a heterogeneous receptor population. For

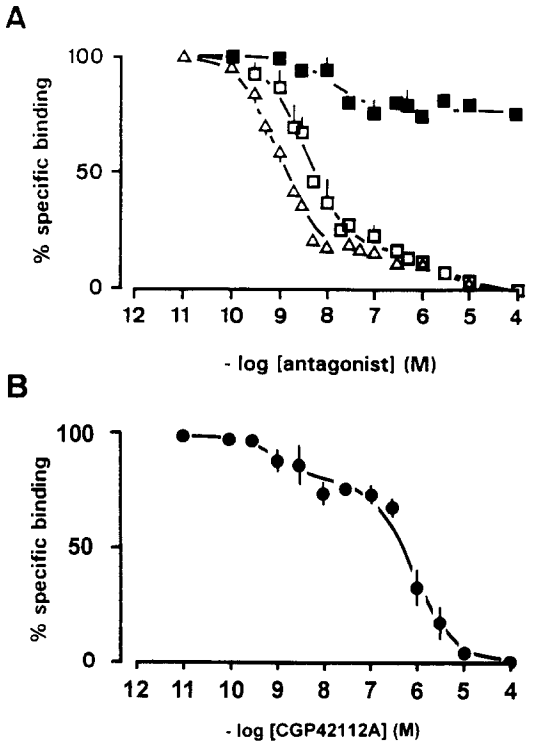

Fig. 3. Effects of non-peptide and peptide angiotensin II antagonists on the specific binding of [^{3}H]angiotensin II to rat portal vein strips. Intact portal vein strips were incubated for 60 min at 37° C with 1 nM [^{3}H]angiotensin II and with increasing concentrations of: (A) DuP 532 (△), losartan (□) and PD 123319 (■), (B) CGP 42112A (●). The values are expressed as a percentage of the maximal specific binding obtained in the absence of unlabelled drugs and are means ± S.E.M. of three to six experiments, each carried out in duplicate.

Table 2
Inhibition of 1 nM [^{3}H]angiotensin II binding to portal vein strips and membranes by non-peptide and peptide antagonists when considering binding to two sites to constitute 75% and 25% of the maximal binding

Antagonists	K_i (nM)		n Hill	n
	75% of binding	25% of binding		
Strips				
Losartan	2.45 ± 0.04	2056 ± 231	0.62 ± 0.08	3
DuP 532	0.66 ± 0.06	3940 ± 481	0.79 ± 0.11	4
PD 123319	ND	6.42 ± 0.24	–	3
CGP 42112A	3854 ± 120	2.46 ± 0.28	0.38 ± 0.04	4
Membranes				
Losartan	11.21 ± 0.04	2930 ± 110	0.50 ± 0.03	4
DuP 532	0.41 ± 0.03	860 ± 50	0.34 ± 0.12	6
PD 123319	ND	2.19 ± 0.21	–	4
CGP 42112A	532 ± 70	0.72 ± 0.30	0.58 ± 0.10	3

Non-specific binding was approximately 15% of total binding. n = number of experiments. Each value is the mean ± S.E.M. of three to six experiments, each carried out in duplicate. ND: not detectable.

losartan and DuP 532, the analysis of the competition experiments evidenced binding at two sites with the proportions of approximately 75% and 25% of binding at 1 nM [^{3}H]angiotensin II (Table 2). In addition, evidence for a two-site model was also obtained with isolated portal vein membranes with high-affinity and low-affinity components accounting for about 75% and 25% of the total binding capacity, respectively (Table 2).

The selective non-peptide antagonist of angiotensin AT_2 receptors, PD 123319, at low concentrations (0.1 nM to 0.1 μM) inhibited only 25% of the [^{3}H]angiotensin II binding in both portal vein strips and portal vein membranes (Fig. 3A and Table 2). The remaining 75% of the sites were not inhibited even by 1000-fold higher concentrations. Therefore, in order to characterize the angiotensin AT_1 receptor in vascular portal vein strips, competition binding experiments were carried out in the presence of 3 μM PD 123319, a concentration that would block all angiotensin AT_2 receptors. Under these conditions, the remaining binding sites were inhibited in a monophasic manner by losartan with a K_i value of 3.09 ± 0.12 nM ($n = 5$). This value is similar to the value obtained for the high-affinity component (Table 2).

In both intact strips and membranes, increasing concentrations (from 0.01 nM to 100 μM) of CGP 42112A, a peptide antagonist which shows a high affinity for angiotensin AT_2 receptors (nM) and a low affinity for angiotensin AT_1 receptors (μM), displaced [^{3}H]angiotensin II in a biphasic manner (Fig. 3B). The analysis of this competition experiment confirmed the binding to two sites with the same proportions of approximately 75% and 25% of binding at 1 nM [^{3}H]angiotensin II (Table 2). Furthermore, in the presence of 0.3 μM CGP 42112A, increasing concentra-

Table 3
Effect of PD 123319 (3 μM) on 1 nM [^{3}H]angiotensin II specific binding to portal vein membranes and strips in the continuous presence of 0.3 μM CGP 42112A

	Strips dpm·mg^{-1} wet weight	Membranes dpm·mg^{-1} protein
CGP 42112A	2054 ± 128	2300 ± 114
CGP 42112A + PD123319	2211 ± 121	2436 ± 90

Non-specific binding was less than 150 dpm/0.1 mg protein (membranes) and 300 dpm/mg protein wet weight (strips). Each value is the mean ± S.E.M. of four experiments, each carried out in duplicate.

tions of PD 123319 (0.01 nM to 100 μM) failed to inhibit [^{3}H]angiotensin II binding in both portal vein strips and portal vein membranes (Table 3).

These results strongly suggest the presence of both angiotensin AT_2 (25% of binding) and angiotensin AT_1 (75% of binding) receptors in portal vein smooth muscle.

3.3. Effects of GTP-γ-S and fluoroaluminates on [^{3}H]angiotensin II binding to membranes and intact strips of portal vein smooth muscle

Extracellular combination of aluminium and fluoride is believed to stimulate G-proteins by mimicking the effects of internal GTP-γ-S on the α subunit (Cockcroft, 1987). The effects of increasing concentrations of GTP-γ-S (10 nM to 0.5 mM) and NaF (1 μM to 100 mM) in the presence of 10 μM $AlCl_3$ on [^{3}H]angiotensin II binding were assessed with membranes and strips, respectively. In each case, the binding was inhibited by 80% with IC_{50} values of 85 ± 20 nM ($n = 3$) for GTP-γ-S and 2.1 ± 0.3 mM ($n = 3$) for fluoroaluminates. This similarity of effect justified the use of fluoroaluminates in the following experiments with strips. In the presence of 3 μM PD 123319 to block angiotensin AT_2 receptors, [^{3}H]angiotensin II binding in intact strips was completely inhibited by fluoroaluminates with an IC_{50} value of 2.7 ± 0.6 mM ($n = 4$; Fig. 4). To elucidate the mode of binding action of NaF, Scatchard analysis of [^{3}H]angiotensin II binding was carried out in the presence or absence of 10 mM NaF (plus 10 μM $AlCl_3$). Fluoroaluminates behave as non-competitive inhibitors (Fig. 5A), decreasing the B_{max} value from 17.7 ± 1.6 fmol · mg^{-1} wet weight in control to 5.3 ± 0.7 fmol · mg^{-1} wet weight in the presence of fluoroaluminates ($n = 4$). In addition, the K_d value was slightly decreased from 2.1 ± 0.3 nM in control to 1.7 ± 0.5 nM in the presence of fluoroaluminates. This variation was not significant ($n = 4$; $P > 0.05$). Fig. 5B shows that fluoroaluminates accelerated the kinetics of dissociation of the [^{3}H]angiotensin II-receptor complex. The rate constant of dissociation increased from 0.019 ± 0.010 min^{-1} in control to 0.070 ± 0.020 min^{-1} in the presence of fluoroaluminates

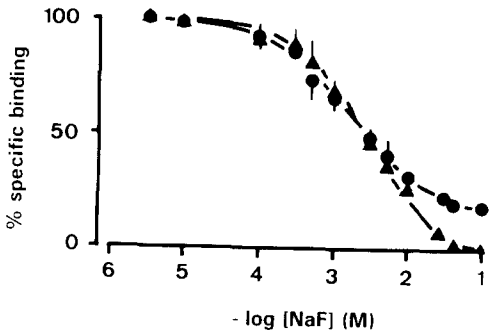

Fig. 4. Effects of fluoroaluminates on the specific binding of [^{3}H]angiotensin II to rat portal vein strips. Intact portal vein strips were incubated for 60 min at 37° C with 1 nM [^{3}H]angiotensin II and with increasing concentrations of NaF (plus 10 μM $AlCl_3$) in the absence (●) or in the presence of 3 μM PD 123319 (▲). The values are expressed as a percentage of the maximal specific binding obtained in the absence of unlabelled drugs and are means ± S.E.M. of three to four experiments, each carried out in duplicate.

($n = 4$, $P < 0.05$). These observations suggest that fluoroaluminates may transform high-affinity [^{3}H]angiotensin II binding sites into low-affinity angiotensin II binding sites.

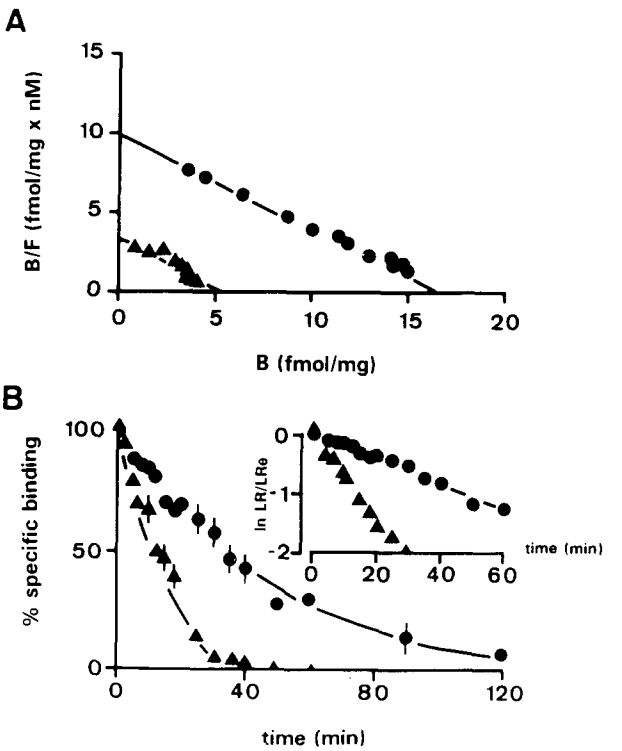

Fig. 5. Effects of fluoroaluminates on the specific binding of [^{3}H]angiotensin II to rat portal vein strips. (A) Scatchard plots obtained in the absence (●) and presence of 10 mM NaF plus 10 μM $AlCl_3$ (▲). (B) Dissociation kinetics of [^{3}H]angiotensin II binding. After equilibration had been reached, dissociation was initiated by addition of 10 μM unlabelled angiotensin II, in the absence (●) or in the presence of 10 mM NaF plus 10 μM $AlCl_3$ (▲). Inset, data plotted as a first-order representation of the time course of dissociation. Each value is the mean ± S.E.M. of four experiments, each carried out in duplicate.

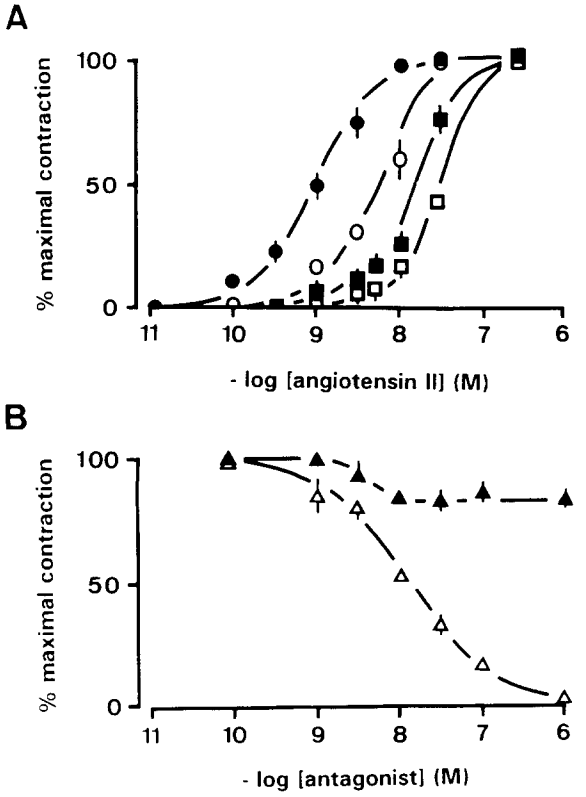

Fig. 6. Effects of non-peptide angiotensin II antagonists on the contractile response to angiotensin II in intact strips from rat portal vein. (A) Concentration-response curves for angiotensin II in control conditions (●) and in the presence of 10^{-8} M (○), 3.10^{-8} M (■ and 10^{-7} M (□) losartan. (B) Effect of losartan (△) and PD 123319 (▲) on the angiotensin II-induced contraction. Contractions are expressed as a percentage of the maximal contraction induced by 10 nM angiotensin II and are shown as means ± S.E.M. of four experiments.

Similar experiments performed with GTP-γ-S and membranes confirmed these results, particularly on the kinetics of dissociation. The rate constant of dissociation increased from 0.018 ± 0.01 min^{-1} in control to 0.080 ± 0.02 min^{-1} in the presence of 0.1 mM GTP-γ-S ($n = 4$, $P < 0.05$).

3.4. Contraction studies

Angiotensin II produced concentration-dependent contractions in isolated strips from rat portal vein, the maximal effect being obtained at 10 nM (Fig. 6A). Losartan revealed a competition antagonism in this model since the concentration-contraction curve for angiotensin II was shifted to the right in a parallel manner, and no depression of the maximal contraction occurred in the presence of 10 nM, 30 nM or 100 nM losartan ($n = 4$). Analysis according to the method of Arunlakshana and Schild (1959) revealed a pA_2 of 8.8 with a slope of 0.9, which is not significantly different from unity.

Fig. 6B shows that losartan inhibited completely the angiotensin II-induced response in a concentration-dependent manner with an IC_{50} value of 13 nM ($n = 4$). In contrast, PD123319 inhibited only 20% of the angiotensin II-induced contraction ($n = 4$). Thus, contraction experiments provide additional evidence for the existence of functional angiotensin AT_1 and AT_2 receptors in portal vein myocytes.

4. Discussion

In the present work, we show the existence of both angiotensin AT_1 and AT_2 receptors in membranes and intact strips of portal vein smooth muscle. These receptors had a high affinity for angiotensin II and the peptide antagonist [Sar^1,Ile^8]angiotensin II (in the nM range). The two types of binding sites were identified by the non-peptide angiotensin AT_2 (PD 123319), angiotensin AT_1 (losartan and DuP 532) and peptide AT_2 (CGP 42112A) receptor antagonists. Evidence supporting this proposal is the following: (1) analysis of the displacement curves for losartan and DuP 532 evidenced high-affinity and low-affinity components accounting for 75% and 25% of the total [^{3}H]angiotensin II binding capacity, respectively; (2) PD 123319 bound with a high affinity to only one binding site population and was not able to inhibit more than 30% of the total [^{3}H]angiotensin II binding; (3) in the presence of 10 μM PD 123319, the low affinity binding component for both losartan and DuP 532 was no longer detectable, and the displacement curves for the angiotensin AT_1 receptor antagonists became monophasic; (4) analysis of the displacement curves for CGP 42112A evidenced that CGP 42112A had high affinity (nM) for the minor site (25%) and low affinity (μM) for the major site (75%); (5) in the presence of CGP 42112A increasing concentrations of PD 123319 failed to inhibit the [^{3}H]angiotensin II binding. These results strongly suggest that 75% of the saturable specific binding sites preferentially binding losartan or DuP 532 are angiotensin AT_1 receptors whereas 25% of specific binding sites selectively blocked by PD123319 or CGP 42112A are angiotensin AT_2 receptors. The existence of two subtypes of angiotensin II receptors has been previously reported in several mammalian tissues such as brain (Chang et al., 1990; Gehlert et al., 1990), heart (Feolde et al., 1993), adrenal gland (Montiel et al., 1993) and myometrium (Cox et al., 1993) but not in rat vascular smooth muscles (Timmermans et al., 1993).

Douglas and Hopfer (1994) have recently proposed the existence of pharmacologically defined AT_{1a} and AT_{1b} receptors in adult rat kidney. According to this proposal, the most abundant site (AT_{1a}) has a high affinity for losartan (nM) and a low affinity for PD 123319 (μM) and CGP 42112A (mM). Conversely, the least abundant site (AT_{1b}) displays a high affinity for

PD 123319 (nM) and a low affinity for losartan (μM) and CGP 42112A (μM). Both angiotensin AT_{1a} and angiotensin AT_{1b} receptors are G-protein-coupled. The pharmacological profile of the angiotensin AT_1 receptor in portal vein smooth muscle shows a low affinity for CGP 42112A (μM) and no sensitivity for PD 123319, suggesting that it cannot be classified as AT_{1a} or AT_{1b}. The inhibition of [^{3}H]angiotensin II binding to portal vein strips and membranes by non-peptide and peptide antagonists was more effective with membranes than with strips, except when losartan was used. The molecular characteristics of angiotensin AT_1 and AT_2 receptors could be different in isolated membranes as several components of the environment and transduction pathways may be lost. Nevertheless, the interaction of losartan with angiotensin receptors appears to be more complex than that of other non-peptide angiotensin antagonists. It has been recently proposed that variations in preparative techniques and protocols may lead to changes in the basal ratio of active and inactive angiotensin II receptors. These changes could modulate the angiotensin II-losartan interactions (Robertson et al., 1994).

Previous studies have suggested on the basis of the inhibitory effects of losartan that the contractile response of vascular smooth muscle to angiotensin II is essentially mediated through activation of angiotensin AT_1 receptors (Pörsti et al., 1993; Yazawa et al., 1993; Zhang et al., 1993). Our results clearly show that the angiotensin II-induced contraction in rat portal vein is mediated by activation of both angiotensin AT_1 and AT_2 receptors. The angiotensin II-induced contraction was inhibited by about 20–25% by 10 μM PD 123319, as expected from the PD 123319-induced inhibition of [^{3}H]angiotensin II binding. Moreover, the concentration-contraction curve for angiotensin II was shifted to the right in the presence of increasing concentrations of losartan without a reduction in the maximal response, suggesting a competitive antagonism at the level of the angiotensin AT_1 receptors. This result is in good agreement with the results of [^{3}H]angiotensin II binding experiments in which high concentrations of losartan completely inhibited the radioligand binding to portal vein membranes. Therefore, these findings indicate that results obtained with losartan alone are not sufficient reason to discard the existence of functional angiotensin AT_2 receptors in vascular smooth muscle.

Angiotensin AT_1 receptors have been reported to be functionally coupled to phospholipase C through G-proteins, as evidenced by the increased formation of inositol phosphates after exposure to angiotensin II (Guillon et al., 1988; Feolde et al., 1993). However, stimulation of Ca^{2+} channels in guinea pig portal vein myocytes has been shown to be mediated by a G-protein that is insensitive to both pertussis toxin and cholera toxin (Ohya and Sperelakis, 1991). Our data indicate that only the angiotensin AT_1 receptors interact with G-proteins in rat portal vein smooth muscle. Activation of G-protein by GTP-γ-S or fluoroaluminates inhibited about 80% of the [^{3}H]angiotensin II binding, the remaining binding component being removed by the addition of PD 123319. Furthermore, Scatchard analysis revealed a 80% decrease in the maximal binding capacity without there being a significant variation in the K_d value. The kinetics of dissociation of the [^{3}H]angiotensin II-receptor complex were accelerated in the presence of GTP-γ-S or fluoroaluminates, suggesting that GTP-γ-S and fluoroaluminates transformed high-affinity [^{3}H]angiotensin II binding sites into low-affinity binding sites with a faster rate of dissociation. In general, activation of G-proteins by GTP analogs results in a reduction in affinity for their specific radioligands (Dohlman et al., 1987; Bottari et al., 1991). Our data thus show that separation of angiotensin AT_1 and AT_2 receptors on the basis of differences in [^{3}H]angiotensin II binding properties and contractile responses is also relevant with regard to signal transduction. Therefore, it is likely that in rat portal vein smooth muscle angiotensin II mediates some of its contractile effects through G-protein-independent mechanisms.

The physiological role of angiotensin II binding to angiotensin AT_2 receptors is unknown. Nevertheless, it has been observed that in rodents (Tsutsumi et al., 1991; Millan et al., 1991), angiotensin AT_2 receptors are transiently expressed at up to 10-fold higher levels than angiotensin AT_1 receptors in specific stages of fetal development, thus suggesting a role in the control of proliferation and differentiation. As growth factors can regulate smooth muscle contractility, via tyrosine-kinase pathways, in a number of vascular and intestinal smooth muscle preparations from a wide spectrum of species (Hollenberg, 1994), it could be proposed that angiotensin II might trigger signal transduction pathways similar to those activated by growth factors. Further studies are required to determine whether the angiotensin AT_2 activation can be correlated with a tyrosine-kinase inhibitor-sensitive increase in phosphotyrosyl proteins or with the presence of inhibitor-sensitive tyrosine kinase activity in tissue extracts.

In conclusion, the contractile response of rat portal vein smooth muscle to angiotensin II is mediated through activation of both angiotensin AT_1 and AT_2 receptors. The transduction pathways involve both G-protein-dependent and -independent mechanisms.

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